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SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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AN IMMUNIZING STRAIN OF THE VIRUS OF POLIOMYELITIS.

By SIMON FLEXNER, M.D., and HAROLD L. AMOSS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, December 21, 1923.)

No means are known by which a strain of the virus of poliomyelitis, active for monkeys, can be attenuated artificially. All human strains hitherto described, once they have been adapted by implantation to the monkey, display high virulence. A certain number of strains, because of inherent low potency, fails to become adapted at all. These latter strains produce on injection no appreciable effect and leave the monkeys as subject to inoculation with a virulent strain as previously untreated animals. Thus up to the present time, two varieties only of the virus of poliomyelitis have become known: one too weak to excite any reaction, and the other so strong as to induce severe paralytic effects in the monkey.

Recently we have encountered a third variety. Ever since our experimental studies on poliomyelitis were begun in 1909, [1–4] we have been on the lookout for a strain of the virus which would be mildly effective in the monkey. Apparently such a strain has now come into our hands. It is of some interest to learn that the human case from which it was derived was unusually severe clinically, and exhibits histologically lesions of exceptional intensity and acuteness.[5]

Source of the Virus.

The patient, a child 5 years of age, from whom the specimen was obtained, was taken ill on Oct. 29, 1921, and died on Nov. 3. The symptoms were

[1] Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1909, liii, 1639.

[2] Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1910, liv, 1780.

[3] Flexner, S., and Lewis, P. A., *J. Exp. Med.*, 1910, xii, 227.

[4] Flexner, S., and Clark, P. F., *J. Am. Med. Assn.*, 1911, lvii, 1685.

[5] We are indebted for the specimens of the nervous system of the case to the interest of Dr. George Draper of New York, and the cooperation of Dr. N. C. Russell and Dr. Perkins Arnold of Buffalo.

at first indefinite; there had been vomiting and anorexia. On the 2nd day of the illness, slight rigidity of the neck muscles and difficulty of swallowing were noted. Paralysis of the soft palate attended by nasal regurgitation developed, and was followed by increasing signs of bulbar involvement. Death occurred from respiratory paralysis.

EXPERIMENTAL.

The brain tissue as received in 50 per cent glycerol was in large sections. As no special precautions could be exercised in removing the brain at the necropsy, bacterial contamination was inevitable. The tissues were somewhat discolored and had a slightly putrefactive odor on their receipt. Test suspensions in agar Petri plates showed innumerable colonies of bacteria.

The large sections were cut up and placed in 50 per cent sterile glycerol. This device did not lead in a few days to any material reduction in the bacteria. Resort was therefore had to phenolation,[2, 3] as this means had previously been found effective against somewhat less degrees of bacterial contamination. The method consisted in immersing thin sections of the brain and spinal cord in abundant 0.5 per cent phenol for 24 hours, removing them for culturing, and again placing in 50 per cent glycerol. As the reduction in bacterial content was not regarded as sufficient, a second immersion in 0.25 per cent phenol was resorted to, and daily cultures were made. On the 4th day, few bacterial colonies developed in the Petri plates, hence animal inoculation was undertaken. Mention should be made of the fact that complete bacterial sterility was not found necessary, as the monkeys readily disposed of the saprophytic bacteria still remaining in the specimens injected. All the monkeys used in the experiments belonged to the species *Macacus rhesus*.[6]

Experiment 1.—Monkey A on Sept. 19, 1922, received intracerebrally 1 cc. of a 10 per cent suspension of the phenolated and glycerolated human material, which on plating showed about 100 colonies of bacteria per cc. No effect was detected until Oct. 1, when excitement, head tremor, and right facial paralysis were observed. These symptoms progressed, and in addition the right deltoid was noted to be weak; ataxia was also present. As on Oct. 6 the condition was stationary, the animal was etherized in order to obtain nervous tissues for further

[6] All inoculations were made in animals anesthetized with ether.

inoculation and for histological examination. The lesions disclosed by the microscope were typical of experimental poliomyelitis. They were of moderate grade of severity and involved the medulla and upper spinal cord chiefly.

Experiment 2.—Monkey B. An immediate transfer from Monkey A to a second *Macacus rhesus* was made. On Oct. 6, 1.5 cc. of a suspension prepared from the site of inoculation and medulla were injected intracerebrally into Monkey B. The suspension was sterile in aerobic culture. The first symptoms were noted on the 12th day; and on the 16th day, head tremor, right facial palsy, with ptosis and weakness of the deltoids were present. The animal was etherized on the 17th day. The lesions in the nervous tissues were those of experimental poliomyelitis.

Experiment 3.—Monkey C received intracerebrally on Oct. 27, 1 cc. of a 10 per cent suspension of the original phenolated and glycerolated human material and intraperitoneally 10 cc. The plated suspension showed about 300 colonies per cc. This animal remained well until Nov. 6, when head tremors and left facial palsy appeared. The next day, 15 cc. of the suspension were given intraperitoneally. On Nov. 8 the animal was ataxic and the right deltoid was weak. On Nov. 10 the back muscles were weak. The animal was etherized. The nervous tissues showed characteristic lesions of experimental poliomyelitis.

This experiment was repeated on three monkeys which were, however, permitted to recover and were subsequently tested with a potent M.A. strain of the virus of poliomyelitis for resistance. While the control animal developed paralysis and succumbed in the usual manner, the animals which developed symptoms from the inoculation of the Buffalo strain of human virus entirely resisted the inoculation. The fact should be mentioned that one monkey developed aphonia which became complete, then gradually receded, as other symptoms abated, until the voice was quite regained.

Experiment 4.—Monkey D. Nov. 22, 1922. Received intracerebrally 1 cc. and intraperitoneally 15 cc. of a 10 per cent suspension of the brain and spinal cord of Monkey C. No symptoms arose until Nov. 15, when the animal was observed to be unduly excitable. The next day ataxia and general tremors, including slight convulsive seizures, were present. As no progression of the symptoms took place, 15 cc. of the suspension were injected intraperitoneally. No effect was produced by the reinjection. The symptoms gradually abated and in a few days the animal was again apparently normal.

This animal was allowed to recover, after which it was (as in the previous examples) submitted to a test for immunity. On Dec. 18 a control *Macacus rhesus* and Monkey D were each given intracerebrally 0.5 cc. of a Berkefeld filtrate of a 5 per cent emulsion of M.A. virus. The control was prostrate on the 12th day and succumbed to experimental poliomyelitis. Monkey D on the other hand never showed any symptoms whatever.

Experiment 5.—Monkey E received intracerebrally on Jan. 3, 1923, 1 cc. of a 10 per cent suspension of the glycerolated brain and spinal cord of Monkey A. Ataxia, head tremors, and weakness of deltoid and back muscles were present

on Jan. 16. The legs became paralyzed on Jan. 18, on which date the animal was etherized. The lesions revealed by the microscope were typical of experimental poliomyelitis.

A mate to this animal, Monkey F, inoculated on the same day as Monkey E, but having received also 15 cc. of the suspension intraperitoneally, showed slighter symptoms and recovered.

These instances represent the second passage, and the next instances the third passage of the virus. As will be noted in the third passage, one monkey developed severe symptoms and two other monkeys much slighter symptoms of experimental poliomyelitis.

Experiment 7.—Monkey G. Sept. 29, 1923. Received intracerebrally 1 cc. of a 10 per cent suspension derived from Monkey E. On Oct. 15 the animal was excitable and tired easily. Reinjected with 1 cc. intracerebrally and 15 cc. intraperitoneally. No appreciable effect was produced by this inoculation. It was repeated on Oct. 23, without immediate result. But on Oct. 28, the animal was tremulous, showed ptosis, right facial palsy, and weakness of the back muscles. On Nov. 1 the monkey was etherized. The microscopic examination showed pronounced perivascular infiltrative lesions in the medulla and spinal cord.

Monkeys H and I of this series developed tremors, ataxia, and deltoid and leg paralyses. They were permitted to recover. The animal which showed a degree of leg involvement preventing standing, subsequently recovered with a trace, only detectable in its movements, of the original extensive paralysis.

DISCUSSION.

The preceding experiments present certain new facts regarding the virus of poliomyelitis. They show, apparently for the first time, that strains of the virus exist in nature which under some circumstances induce paralytic and lesser effects in monkeys, the inclination of which is toward amelioration terminating in recovery, instead of, as had been observed in the past, intensification leading to death. This latter consideration may in time come to have practical significance.

The qualities which distinguish the attenuated strain of the virus raise the question whether the mild grade of activity is an inherent property, or whether it is a product of the mild putrefactive changes taking place in the human tissues. No answer can be returned at present to this question, which, however, may not prove entirely beyond experimental determination.

It has been ascertained by experimental tests that the modified form of poliomyelitis through which the inoculated monkeys pass and from which they recover, leaves them protected against the action of a highly virulent strain of the virus of poliomyelitis. The Rockefeller Institute possesses several such potent strains, which are so active that fractions of a cubic centimeter of a Berkefeld filtrate of a 5 per cent suspension, injected intracerebrally, induce with certainty in 6 or 7 days paralysis leading to death. The several monkeys which had shown symptoms of mild poliomyelitis following injections of the attenuated virus and which were permitted to recover, were subsequently inoculated intracerebrally with a highly virulent (revived M. A. strain)[7,8] of the virus at the same time with controls. The results were consistent: the former never showed any appreciable symptoms, while the latter developed paralysis, usually by the 7th day, and succumbed by the 14th day.

The mild or attenuated strain of the virus seems to be of a fixed degree of potency, having neither increased nor decreased perceptibly in the 2 years during which it has been under study. Unless the virus was modified by the putrefactive process occurring in the original human brain and cord, it is obvious that wide disparity exists between the effects induced by the strain in the human being from whom it was derived and the monkeys into which it was inoculated. The former shows very severe, acute lesions in the spinal cord and medulla to which the death is attributable; the latter shows relatively mild lesions from which recovery, either complete or nearly complete, tends to take place.

The lesions present in the monkeys which were sacrificed explain not only the recovery in those allowed to go on to completion of the pathological process, but they afford an indication of the manner and degree of the resolution process. In the first place, the medulla is less often and less extensively affected than is the rule with the more potent virus. The severer lesions are found in the spinal cord and intervertebral ganglia. The spinal cord may show a degree of neuronophagocytosis, thus accounting for such slight residual paralysis

[7] Flexner, S., Clark, P. F., and Amoss, H. L., *J. Exp. Med.*, 1914, xix, 195.
[8] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1924, xxxix, 191.

as sometimes occurs. But the chief interest centers about the extensive perivascular infiltrations and the milder interstitial infiltrations, which in time seem capable of undergoing complete resolution with which is connected the restitution of function taking place in paralyzed members.

SUMMARY.

In this communication we have described a strain of the virus of poliomyelitis derived from a rapidly fatal human case, which exhibits mild degrees of infective power and marked degrees of protective effect for the monkey. While *Macacus rhesus* displays perceptible differences in suceptibility to the attenuated virus, nearly all individuals respond to the inoculation and none succumbs to the infection induced. As compared with previously described examples of experimental poliomyelitis, the modified disease described in this paper is distinguished by its relatively benign nature and its tendency to end in recovery rather than, as with the earlier observed instances, in death.

EXPERIMENTS ON ANAPHYLAXIS TO AZOPROTEINS.*

By K. LANDSTEINER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, December 26, 1923.)

It is known from previous work[1] that it is possible to produce immune bodies which act specifically on various simple chemical groups. The antibodies referred to developed in rabbits after the injection of chemical compounds of certain simple substances with proteins. The immune sera caused precipitation when added to the antigens employed. In order to demonstrate the specificity of reaction against these simple groups, different proteins were used for immunization and for the tests *in vitro*. This was done so as to exclude such reactions as are dependent upon the quality of the injected protein itself.

It was an obvious task to ascertain whether similar results could be obtained by the anaphylactic method. If this proved the case, it should be possible to discover whether the simple substances contained in the antigen will suffice to produce anaphylactic shock or antianaphylaxis. The relation of an investigation of this character to the problem of drug allergy is evident.[2] Since animals sensitized with antigens containing various proteins can be tested with one and the same substance by means of the method, it might also be useful in comparing the antigenic activity of different proteins.

The investigations have been restricted by a technical difficulty, *i.e.* the toxicity of the solutions when injected intravenously. The method employed for their preparation, which is described below, failed to give constant results in this respect. Hence the toxicity of the solutions had to be tested on normal animals before the solutions could be used on sensitized animals.

* Eighteenth paper on antigens.

[1] Landsteiner, K., and Lampl, H., *Biochem. Z.*, 1918, lxxxvi, 343; 1920, civ, 280.

[2] For the discussion of this question see Wells, H. G., *Physiol. Rev.*, 1921, i, 44. Coca, A. F., in Tice, F., Practice of medicine, New York, 1920, i, 107. Doerr, R., *Schweiz. med. Woch.*, 1921, ii, 937.

The methods, both for preparing the test solutions and for the carrying out of the animal experiments, have not been greatly varied; further efforts may lead to their improvement.

The numerous communications dealing with attempts to induce hypersensitiveness by means of simple chemical substances or by passive treatment with serum of hypersensitive human beings need not be referred to, but mention should be made of certain experiments with artificially altered proteins.[3] From them it appears that animals can be sensitized with proteins modified sufficiently to impair their species specificity (xanthoprotein, iodoprotein). The animals reacted with the protein used for the sensitization and usually with unchanged protein of the same animal, and furthermore in some of the reported experiments with other proteins which had been altered in a similar way as the sensitizing protein. As a rule, the experiments did not give as regular results as in the case of anaphylactic tests with unchanged proteins.

EXPERIMENTAL.[4]

For the sensitizing injections the solution was prepared as follows: 4 gm. sodium para-arsanilate (atoxyl) was diazotized in the usual way,[5] and the solution was added to a mixture of 400 cc. of horse serum and 100 cc. of 10 per cent sodium carbonate. After standing for 5 minutes at room temperature, the azo-compound of the protein was precipitated by adding sufficient hydrochloric acid to bring about a faintly acid reaction on Congo red paper. Foaming can be prevented by the addition of octyl alcohol. The precipitate was filtered off and dissolved in water by means of a weak solution of sodium hydroxide. The resulting liquid, which should give a neutral reaction to litmus, was precipitated by alcohol, the precipitate collected, and the precipitation with alcohol repeated. Then the yellow precipitate was dissolved as before and precipitated by hydrochloric acid. After redissolving and neutralizing the brown solution was adjusted to a content of about 1 per cent sodium chloride and 0.5 per cent phenol and

[3] Wells, H. G., *J. Infect. Dis.*, 1908, v, 467. Pick, E. P., and Yamanouchi, T., *Z. Immunitätsforsch., Orig.*, 1908–09, i, 676. Schittenhelm, A., and Ströbel, H., *Z. exp. Path. u. Therap.*, 1912, xi, 102. *Cf.* Freund, H., *Biochem. Z.*, 1909, xx, 503. von Dungern, and Hirschfeld, *Z. Immunitätsforsch., Orig.*, 1911, xi, 557.

[4] A short report of a part of the experiments appeared in an article by Landsteiner, K., *Kon. Akad. Wetensch. Amsterdam*, 1922, xxxi, 54.

[5] Ehrlich, P., and Bertheim, A., *Ber. chem. Ges.*, 1907, xl, 3292. Jacobs, W. A., and Heidelberger, M., *J. Am. Chem. Soc.*, 1921, xliii, 1632.

filtered through gauze. The volume of the solution being 360 cc., it contained, on a rough estimation 7 per cent of the coupled protein.

Guinea pigs weighing 200 to 260 gm. received four injections of the solution at weekly intervals; the animals were given 1 cc. for each of the first three injections, and 2 cc. in the fourth injection. In previous experiments[4] the animals were given only three injections.

The solution used for reinjection in most of the experiments[6] was prepared from chicken serum in the same manner as the horse azoprotein, using 2 gm. of the *p*-arsanilic salt for every 100 cc. of serum plus 50 cc. of 10 per cent sodium carbonate and coupling for 10 minutes at room temperature. No phenol was added. The solution was precipitated three times with alcohol and then freed from suspended particles by careful centrifugation. The protein content was about 5.5 per cent.

As a rule, the sensitization was well tolerated, and the animals gained in weight. The reinjection was made intravenously 4 to 6 weeks after the last sensitizing injection at which time they weighed from 350 to 520 gm. Normal animals of corresponding weight were used as controls. Analogous azoproteins were prepared with other azo-components, but on intravenous injection were found to be too toxic for use. The following results were obtained.

Experiment 1.[4]—Fourteen sensitized animals were reinjected with from 1 to 2 cc. of azoprotein solution. Five died within 3 to 7 minutes; three others had severe, and five, slight anaphylactic symptoms. Nine normal animals receiving corresponding quantities of the azoprotein solution, showed no distinct symptoms.

Experiment 2.—This experiment was carried out in the same way. The results follow.

Normal guinea pigs.	Weight.	Quantity of azoprotein solution injected.	Subsequent change in body temperature.	Symptoms.
	gm.	*cc.*	°C.	
1	350	2.0	−2.6	Somewhat weak.
2	350	1.5	−2.0	No marked symptoms.
3	311	1.5	−1.5	Somewhat weak.
4	377	2.0	−0.4	Slight coughing.
5	381	1.5	+1.7	No marked symptoms.
6	363	1.5	+0.6	" " "
7	402	1.5	−0.6	" " "
8	365	1.5	−1.1	" " "

[6] In the former experiments the preparation was made according to Landsteiner, K., *Biochem. Z.*, 1918, lxxxvi, 362. 4 gm. atoxyl were used and coupled for 30 minutes.

Sensitized guinea pigs.	Weight.	Quantity of azoprotein solution injected.	Subsequent change in body temperature.	Symptoms.
	gm.	*cc.*	°C.	
9	338	1.0		Death in 5 min.
10	396	1.0	−2.4	Moderate symptoms.
11	478	1.5		Death in 4 min.
12	401	1.5	−0.7	No marked symptoms.
13	364	1.0		Death in 15 min.
14	390	1.5	−1.4	Slight symptoms.
15	392	1.5	−6.0	Death in 3½ hrs.
16	395	1.5	−7.2	Severe symptoms.

Very slight symptoms such as scratching and slight jerking are not mentioned in the tables.

The symptoms were typical anaphylactic ones. Of the eight sensitized animals, four died, one had severe, one, moderate symptoms, while none of the eight control animals showed marked symptoms. In the animals which succumbed, the lungs were distended, mostly showing a few hemorrhages. The changes in temperature reported are those occurring during the first hour after injection.

Experiment 3.—A number of the animals sensitized like those of Experiment 2 received injections intravenously, first of azo-compounds made by coupling diazotized amino acids with aromatic oxy acids and 1 hour afterwards of chicken azoprotein into the jugular vein of the other side. The azo-compounds were prepared according to the method of Pauly[7] and precipitated by acidifying. The precipitate was washed with water and alcohol. The compound of meta-amino-benzol-sulfonic acid was precipitated by adding alcohol and sodium chloride. From the dry substances, solutions were made in saline containing 0.25 per cent of the azo-compounds. 0.5 cc. of the solutions was injected. For the second injection, 1.5 cc. of the azoprotein solution were used.

[7] Pauly, H., *Z. physiol. Chem.*, 1915, xciv, 284. See Landsteiner, K., *Biochem. Z.*, 1920, civ, 280.

Sensitized guinea pigs.	Weight.	Azo-compound injected.	Subsequent change in body temperature.	Symptoms.
	gm.		°C.	
17	402	*p*-arsanilic acid + tyrosine.	+0.6	No marked symptoms.
18	359	" " "	+0.2	" " "
19	386	" " "	+1.6	" " "
20	446	" " "	+0.4	" " "
21	405	" " "	−0.4	" " "
22	407	" " "	−1.8	" " "
23	417	" " "	−1.3	" " "
24	374	*p*-arsanilic acid + *p*-oxy-benzoic acid.	+0.3	" " "
25	338	" " "	+0.1	" " "
26	505	" " "	−0.8	" " "
27	415	*m*-amino-benzol-sulfonic acid + tyrosine.		Death in 20 min.
28	392	" " "	+0.8	No marked symptoms.
29	433	" " "	−1.3	" " "
30	436	" " "		Death in 4 hrs.
31	403	" " "	−2.2	Moderate symptoms.
32	424	" " "	−4.3	Severe symptoms.
33	357	2-amino-*p*-toluic acid + *p*-oxy-benzoic acid.	−0.5	No marked symptoms.
34	380	" " "		Death after some hours during night.
35	517	" " "		Death in 31 min.

Slight symptoms, such as scratching and slight jerking, occurred in almost all animals and are not reported in the table. None of the nine animals previously injected with the derivatives of *p*-arsanilic acid showed marked symptoms, while nine injected with other "heterologous" compounds reacted almost in the same proportion as the simply sensitized one. Four of these animals died; one showed severe, and one moderate symptoms. Death, however, did not occur as soon as in the previous experiments.

The injection of the various simple azo-compounds failed to give rise to distinct symptoms in sensitized animals. Even 1 cc. of 2.5 or 5 per cent solutions of the azo-compound of *p*-arsanilic acid and tyrosine, and 1 cc. of a 2 per cent solution of sodium para-arsanilate were without effect when injected intravenously.

SUMMARY.

From the experiments which have been reported, it follows that animals sensitized with one azoprotein react not only to the antigen used for the sensitization, but also to other azoproteins made up from the same simple azo-compounds and another protein. Although the specificity of the reaction has not yet been tested with various azo-components, its actual existence can reasonably be assumed on the basis of the phenomena observed in precipitation reactions.[1] The sensitization is brought about with less facility than sensitization against the usual antigens and the effects are not uniform. Still, after sufficient treatment, 40 per cent of the animals succumbed with typical anaphylactic symptoms, mostly within a short time while 16 per cent showed severe symptoms.

The experiments show that it is possible to make animals hypersensitive against a simple chemical group like para-arsanilic acid, and from this point of view connection would seem to be established with the phenomena of drug allergy in human beings.[8]

There is an essential difference, however, in that the sensitized animals did not react on injections of simple compounds such as para-amino-phenyl-arsanilic acid and phenyl-4-arsonic-acid-azo-tyrosine uncombined with protein. It remains to be determined whether under changed conditions positive results in this direction can be obtained.[9] For this purpose it seems advisable to make experiments with isolated organs, according to the method of Schultz and Dale. While the simple substances failed to elicit direct reactions, they protected (as was foreseen by Doerr) against a subsequent injection of the active antigen. Similar compounds not containing the arsanilic acid group were considerably less active. The phenomenon is comparable to the inhibition of precipitin reactions already described.[1] Considering the protection as a condition of antianaphylaxis, one would suppose that the simple substances mentioned are fixed by the cells in which the anaphylactic reaction takes place.

It may be concluded that:

[8] Doerr, R., in *Ergebn. Hyg., Bakt., Immunitätsforsch., u. exp. Therap.*, 1922, v, 100.

[9] *Cf.* the results of Swift and Curschmann and others.

1. Animals can be sensitized through injections of one azoprotein—protein combined with diazotized para-arsanilic acid—against another compound containing the same azo-component but a different protein.

2. Injections of related simple compounds, as for instance para-arsanilic acid and phenyl-4-arsonic-acid-azo-tyrosine did not cause shock in the sensitized animals under the conditions of the present experiments.

3. The simple compounds mentioned and other related substances as well protect against the anaphylactic action of azoprotein, by inducing a state of antianaphylaxis.

THE EFFECT OF X-RAY ON THE SKIN OF VITALLY STAINED WHITE MICE.

Establishment of an X-Ray Unit for the Skin of the Mouse.

By GERTY T. CORI, M.D.

(*From the State Institute for the Study of Malignant Diseases, Buffalo.*)

(Received for publication, December 11, 1923.)

Numerous experiments have been carried out for the purpose of sensitizing the body toward the visible and ultravisible rays of the sun and artificial light sources (Hausmann (1)). Yet little is known about the sensitization of the animal body towards x-ray.

Ellinger (2) injected rats subcutaneously with a 10 per cent solution of thorium nitrate and found that a smaller amount than usual of subsequent radiation led to marked ulceration of the skin. Similar experiments on cancer patients, in which the tumor itself was infiltrated with the thorium salt, led to a disintegration of the tumor after a radiation with one tenth to one half of the erythema dose. Baldwin (3) found that about one half of the x-ray energy that killed normal paramecia was sufficient to kill vitally stained paramecia. He repeated these experiments on metazoa, injecting white rats with trypan blue. Baldwin found that while the unstained rats died within 100 to 120 hours after radiation, the stained animals survived only 60 to 70 hours. He explains the sensitization of the tissues towards x-ray by an absorption of the stain on the surface of the cell nucleus, a phenomenon which occurs, as he says, most actively under the direct influence of an electromagnetic form of energy.

The following experiments were undertaken to investigate whether the skin of vitally stained white mice is more sensitive toward x-ray than the skin of normal mice. In connection with these experiments an attempt was made to establish a unit of x-ray energy for the skin of the mouse, which could be compared with the human erythema dose. Since an x-ray erythema does not develop on the skin of the mouse, the spontaneous total epilation of the radiated area was chosen as a unit.

EXPERIMENTAL.

White, male, full grown mice of a single strain were used exclusively. The animals were kept in pairs in small cages and fed on dog biscuits and water. One of each pair received the injection of the stain, the other served as a control. Trypan blue (Grübler) was chosen for the vital staining. The stain was made up to a concentration of 0.5 per cent with normal saline. The mice received a subcutaneous injection of 0.5 cc. of this solution on the morning of the 1st, 3rd, and 5th day. On the afternoon of the 5th day the mice were rayed. The legs were fastened with clamps so that the animal could not move and the body was covered with lead sheets, leaving free a field of 1.0 × 1.5 cm. on the left hind part of the back. The control mice were rayed in exactly the same way. A Kelly-Koett apparatus with a Coolidge tube was used. A radiation of 4 milliamperes, 95 kilovolts at a skin target distance of 22 cm. with an aluminum filter of 2.5 mm., was given. The human erythema dose using this combination for a field of the same size is 12 minutes. Fifty animals, of which twenty-five were stained and twenty-five served as controls, were radiated. Groups of ten received a radiation of 80, 70, 65, 60, and 50 minutes.

It was found that the reaction of the radiated skin in stained and control mice was not markedly different, the sensitiveness of that of the stained mouse being apparently slightly below that of the control mouse. There was, however, a marked difference in the time at which epilation began after radiation. Data on this point are summarized in Table I.

It can be seen that the epilation occurred earlier in the stained than in the control mice, rayed for the same length of time. The 80 and 70 minute radiation caused in all a skin ulceration which occurred a few days after the beginning of the epilation. The ulcer was subsequently covered with a crust and finally a scar was formed. A new growth of hair in the radiated region never followed. The skin on the ventral part of the trunk opposite to the rayed area showed a complete epilation without ulceration. This area received about 70 per cent of the rays, since the tissues they had to pass were about 1 cm. thick. The 65 minute radiation caused total epilation in the dorsal and incomplete in the ventral field. All five control mice of this group developed a small ulceration on the dorsal skin, while out of the five stained ones, only three had superficial ulcerations. A new growth of hair took place in several instances on the ventral field. The 60 minute radiation caused complete epilation and a

slight superficial ulceration in three out of the five controls and in one out of the five stained mice. The ventral field was unaffected.

The 50 minute radiation caused no epilation either in vitally stained or control mice. Nearly all the mice were kept under observation for 3 months after radiation.

The epilation dose for the normal mouse lies between 50 and 60 minutes with the radiation combination above described. This

TABLE I.

Time interval between radiation and the occurrence of epilation.

80 min. radiation.		70 min. radiation.	
Control mice.	Stained mice.	Control mice.	Stained mice.
days	*days*	*days*	*days*
16	15	18	16
16	10	20	14
18	10	18	11
17	11	20	13
22	14	21	14
Average 17.8	12	19.4	13.6
65 min. radiation.		60 min. radiation.	
18	15	16	16
21	14	23	14
20	12	22	22
17	12	26	21
17	13	24	20
Average 18.6	13.2	22.2	18.6

amount should cause total spontaneous epilation without any ulceration, but the individual variability has to be taken into consideration. Ten mice were radiated for 60 minutes. For four mice this proved the exact epilation dose, while for four it was a dose slightly above the epilation dose, since superficial ulceration was observed. The two other animals developed only incomplete epilation.

The epilation dose for mice is about four to five times more than the human erythema dose, and corresponds closely to that which Wood

and Prime (4) had to apply in order to kill mouse carcinoma outside the body; Seitz and Wintz (5) have shown that in human beings the ratio of erythema dose to carcinoma dose is about 100:120. It has since been found that this does not hold true for every type of human tumor. The argument that huge doses of x-ray are required to destroy mouse carcinoma is obviously fallacious, because the human erythema dose has no significance for the mouse. Since apparently there is a difference in the sensitiveness toward x-rays of the skin in different species, the epilation dose of each species has to be taken as basis and not the human erythema dose. Experiments are in progress to establish the relationship between the epilation dose and the dose which destroys mouse carcinoma *in vivo*.

Kok and Vorlaender (6) determined a skin dose for the mouse. They termed epilation dose the amount of x-rays which made it possible to pull out easily the hair in the radiated region after a certain length of time. This dose amounts to one to two human erythema doses. But the force necessary to pull out hair easily is arbitrary and cannot well be standardized. The authors mentioned state further that much larger doses caused no ulceration of the skin of the mouse. Consequently the epilation dose established in the present work which is based on spontaneous epilation, is much greater than that of Kok and Vorlaender.

SUMMARY.

1. The time interval between radiation and the occurrence of epilation is shorter in mice injected with trypan blue than in normal animals.

2. An x-ray unit defined as that causing total spontaneous epilation on the skin of the mouse is suggested. It corresponds to four to five human erythema doses.

BIBLIOGRAPHY.

1. Hausmann, W., Grundzüge der Lichtbiologie und Lichtpathologie. VIII. Sonderband zu "Strahlentherapie," Berlin and Vienna, 1923.
2. Ellinger, P., Steigerung und Abgrenzung der biologischen Strahlenwirkung, *Fortschr. Geb. Röntgenstrahlen*, 1922, xxx, 174. Ellinger P., and Rapp, Das Thorium als Sensibilisierungsmittel, *Strahlentherapie*, 1923, xv, 851.

3. Baldwin, W. M., A study of the combined action of x-rays and of vital stains upon paramecia, *Biol. Bull.*, 1920, xxxix, 59; The increased absorption of x-rays by vitally stained white rats, *J. Exp. Med.*, 1923, xxxvii, 357.
4. Wood, F. C., and Prime, F., Effect of x-ray on tumors, *J. Cancer Research*, 1919, iv, 49; Die tötliche Roentgenstrahlendoses für Krebszellen, *Strahlentherapie*, 1921, xiii, 620.
5. Seitz, L., and Wintz, H., Grundsätze der Röntgenbestrahlung des Gebärmutterkrebses und des Karzinoms im Allgemeinen. Die Karzinomdosis, *Münch. med. Woch.*, 1918, xliv, 89.
6. Kok, F., and Vorlaender, K., Biologische Versuche über die Wirkung der Bestrahlung auf das Karzinom, *Strahlentherapie*, 1923, xiv, 497.

PENETRATION OF NORMAL MUCOUS MEMBRANES OF THE RABBIT BY TREPONEMA PALLIDUM AND THE INFLUENCE OF THIS MODE OF INFECTION UPON THE COURSE OF THE DISEASE.

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Plates 33 and 34.

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The apparent infrequency of infection, as compared with exposure, has led to the belief that the infectivity of *Treponema pallidum* is much less than that of the great majority of pathogenic organisms, and that infection is not apt to occur unless virulent spirochetes are brought in intimate contact with some skin or mucous surface where there is an abrasion or other lesion. This conception of the infectivity of *Treponema pallidum* is supported, to some extent, by experimental observations, and in principle, is doubtless correct. Still, the great mass of evidence bearing on these points is based on the occurrence or non-occurrence of outspoken manifestations of syphilis and very little is known as to what actually happens among those who are equally exposed but, in the absence of obvious lesions, may never consult the syphilologist. There are, however, many patients in whom a syphilitic infection of obscure origin is eventually detected. Some of these give a history that indicates the previous occurrence of some manifestation of syphilis, while in others the infection appears to have pursued an asymptomatic course for a long or indefinite period of time.

The group of persons in whom syphilis tends to pursue a mild or asymptomatic course is of especial importance from the standpoint of the transmission of the disease, but they are also important in that they show that infections may occur which do not pursue the classical course. At the present time, it is impossible to say how frequent such infections are, or to what they may be attributed.

The resistance of the patient and the virulence of the organism certainly play a part in the occurrence of these conditions, but there is some evidence to show that the circumstances under which the infection is contracted may be of equal importance.

In man, it is a well established fact that infection may take place without the production of a characteristic primary lesion (1), and such cases are frequently observed in rabbits inoculated in the skin. Moreover, Reasoner (2) has shown that with rabbits, the presence of a gross lesion of the genital mucosa is not essential to successful inoculation; that in occasional instances mere contact of *Treponema pallidum* is sufficient to lead to infection, as indicated by the development of a chancre. What happened in other instances could not be determined on account of the lack of suitable diagnostic criteria.

In like manner, experience has shown that many factors may affect the results of experimental inoculations. This fact is clearly brought out in a recent paper by Chesney (3) who studied the influence of such individual factors as sex, age, and site of inoculation, in animals where all other conditions were constant. In fact, it is apparent from these experiments, that the circumstances under which a syphilitic infection is contracted form one of the major conditions that determine the general course of the disease.

In this connection, we wish to report the results of experiments that were carried out with three strains of *Treponema pallidum* to determine whether infection could be produced in any considerable proportion of animals by bringing these organisms in contact with apparently normal mucous membranes, and how such infections compared with those produced by mechanical inoculations of the same organism. The experiments were carried out in the spring of 1921. A preliminary report was published at that time (4) and reference has been made to the experiments on several occasions, but hitherto they have not been reported in full.

EXPERIMENTAL.

Methods and Material.—The results of the application of *Treponema pallidum* to normal mucous membranes were studied in three groups of male rabbits, using the penile mucosa and the conjunctiva as sites of inoculation. The animals were carefully examined prior to in-

oculation in order to make sure that there was no visible lesion of the mucosa in particular, and that there were no lesions in other parts of the body that might give rise to confusion. In like manner, the inguinal and popliteal nodes were examined in order to exclude animals with preexisting abnormalities.

Inoculations were made with testicular emulsions using a dose of 0.05 cc. which was carefully instilled into the conjunctival sac or the sheath. In the first instance, the lower lid of one eye was retracted to form a pouch for the reception of the emulsion, while with genital inoculations the sheath was drawn forward in a similar manner. In each case, manipulations were confined to the skin and the parts were held in position for about 30 seconds after the emulsion had been introduced. Most of the fluid escaped when the parts were released, leaving only a thin film covering the surface of the membranes.

Experiment 1.—In the first experiment, six animals were inoculated in the right eye and on the sheath with the Nichols strain of *Treponema pallidum*. The emulsion used contained three to six spirochetes to the microscopic field (Table I, Group A). The experiment was controlled by six other animals inoculated intracutaneously on the sheath with 0.05 cc. of the same emulsion (Table I, Group B).

Experiment 2.—The second experiment was also carried out with six animals, inoculated in this case with the Zinsser-Hopkins strain of *pallidum*, using an emulsion containing twelve to fifteen spirochetes to the microscopic field. Three of the animals were inoculated in the right eye (Table II, Group A) and three on the sheath (Table II, Group B). There were again six controls, but in this instance the control animals were inoculated in both testicles with 0.2 cc. of the emulsion (Table II, Group C). This was done as a means of establishing a basis for comparison with large series of animals in which this method of inoculation had been used.

Experiment 3.—The third experiment was carried out merely for the purpose of determining whether organisms of low virulence were capable of gaining entrance to the body through normal mucous membranes. Three animals were inoculated in the right eye and sheath with a recently isolated strain of *pallidum*. The emulsion contained twelve to fifteen spirochetes to the microscopic field. No controls were inoculated.

Results.

The results of the experiments cited above may be considered from two standpoints: First, the occurrence of infection, and second,

the character and course of the disease as compared with that produced by various methods of inoculation involving a mechanical introduction of spirochetes into the body.

Occurrence of Infection.—The criteria used for determining infection were: (1) the occurrence of a local lesion in which spirochetes could be demonstrated; (2) enlargement and induration of drainage lymph nodes; (3) general lymphadenitis; (4) the occurrence of metastatic lesions in other parts of the body, especially the scrotum and testicles. The data covering these points in Experiments 1 and 2 are tabulated in Tables I and II, respectively.

By reference to Table I, it will be seen that all of the animals in the first experiment developed a local lesion at one or the other of the two sites of inoculation. In only one instance, however, was there a lesion at both sites (Figs. 1 and 2), while two other animals showed suggestive conditions about the eye with definite lesions of the penile mucosa.

All of the animals also showed definite enlargement and induration of the inguinal lymph nodes (Fig. 3) as well as a general lymphadenitis.

Metastatic lesions occurred in the scrotum (Fig. 3) of all animals and definite lesions were detected in the testicles of four of the six animals.

The time of occurrence of the several groups of lesions is especially interesting. The incubation period of those at the site of inoculation varied between 34 and 108 days, with an average of 54 days in the case of the eye, and 59 days for the sheath, which is much longer than that of primary lesions in control animals. A definite inguinal adenitis was recognized within a few days after inoculation, while enlargement and induration of the popliteal lymph nodes occurred within 3 to 7 weeks. In several instances scrotal lesions were recognized before any abnormality could be detected at the site of inoculation.

It was possible, therefore, to establish the occurrence of an infection in all of the animals of this group on the basis of a lesion at one or the other of the two sites of inoculation, as well as by the occurrence of a local and general lymphadenitis, and metastatic lesions in the testicles or scrotum. It is important to note, however, that in several instances the first lesions to appear were not at the site of

inoculation, and further, that during the prescribed period of observation, none of the animals inoculated in this manner developed generalized lesions other than those of the scrotum and testicles.

The results of the second experiment (Table II) were much the same as those of the first. The one difference to be noted is in the case of Rabbit 1, Group B. This animal developed no lesion at the site of inoculation, but infection was first established by the development of a small cutaneous papule in the scrotum, and later by the occurrence of other lesions in the testicles and scrotum, as well as by enlargement and induration of the inguinal and popliteal nodes.

The third experiment was carried out with a recently isolated strain of *pallidum* whose virulence was comparatively low, and here the results were less obvious than in the two preceding experiments. Only one of the three rabbits developed clearly defined syphilitic lesions at the sites of inoculation. The two others showed no lesion about the eyes but both developed minute and ill defined areas of redness and apparent infiltration in the region of the urinary meatus, which may or may not have been syphilitic. One of these animals showed a papular lesion in the scrotum, and a second papule in the skin near the anus, while the other developed a definite metastatic orchitis, and all three animals showed a moderate but definite enlargement and induration of the inguinal lymph nodes (Fig. 4).

It was possible, therefore, to demonstrate infection in these animals as in the others, although only one of them developed a characteristic lesion at the site of inoculation.

Character and Course of Disease.—In comparing the disease produced in rabbits by entrance of spirochetes through apparently intact mucous membranes with that produced by ordinary methods of mechanical inoculation, the points to be given especial consideration are: (1) the occurrence or non-occurrence of a so called primary lesion, (2) the character of the primary lesion, (3) the occurrence of generalized lesions, and (4) the general course and severity of the disease.

Of twelve animals inoculated by applying a spirochete emulsion to the genital mucosa, seven developed definite lesions at the site of inoculation, but none of these presented the appearance of a typical

TABLE I.

Results of Experiment 1.

Group and No. of animal.		Site of inoculation.	Lesion at site of inoculation.		Lymphadenitis.		Metastatic lesions.		Other generalized lesions.		Severity of disease.	Period of observation.
			Extent.	Time of occurrence.	Satellite.	General.	Testicles.	Scrotum.	Extent.	Character and time of occurrence.		
				days						*days*		*days*
A.	1	Eye.	+++	62								
		Sheath.	++	34	+	+	?	+	−	−	++	108
	2	Eye.	?	−								
		Sheath.	+	35	+	+	?	+	−	−	+	108
	3	Eye.	+++	45								
		Sheath.	−	−	+	+	+	+	−	−	++	101
	4	Eye.	+	45								
		Sheath.	−	−	+	+	+	+	−	−	+	108
	5	Eye.	?	−								
		Sheath.	+	108	+	+	+	+	−	−	+	108
	6	Eye.	−	−								
		Sheath.	+	59	+	+	+	+	−	−	+	108

B. Controls.											
1	Sheath.	++++	13	+	+	++	++++	++++	S. 51	++++	119
2	"	++++	13	+	+	++	++++	++++	" 58	++++	12 mos.
3	"	++	13	+	+	+	−	+	" 66	++	108
4	"	+	13	+	+	−	−	−		+	108
5	"	+	13	+	+	++	−	−		+	108
6	"	++	13	+	+	?	−	++	S. 58; B. 61	+++	

Group A. Animals inoculated on mucous membranes.

Group B. Control animals inoculated intracutaneously on the sheath.

? indicates result or condition uncertain; −, negative; +, positive where used with reference to lymph nodes, elsewhere indicates also slight or mild affection; ++, moderate affection; +++, well developed lesions or severe disease; ++++, marked lesions or very severe disease. B., bone; E., eye; M. M., mucous membranes; S., skin.

TABLE II.

Results of Experiment 2.

Group and site of inoculation.	No. of animal.	Lesion at site of inoculation.		Lymphadenitis.		Metastatic lesions.		Other generalized lesions.		Severity of disease.	Period of observation.
		Extent.	Time of occurrence.	Satellite.	General.	Testicles.	Scrotum.	Extent.	Character and time of occurrence.		
			days						*days*		*days*
A. Eye.	1	+	45		+	+	+	−	−	+	109
	2	+	38		+	+	+	+	S. 95	+	102
	3	+	59		+	++	−	?	?	+	11 mos.
B. Sheath	1	?		+	+	+	+	−	−	+	102
	2	+	54	+	+	+	+	−	−	+	102
	3	++	33	+	+	+	+	−	−	+	109
C. Testicles	1	++++	16		+			−	−	+	104
	2	++++	16		+			+	S. and M.M. 99	++	104
	3	++++	16		+			++	B. 76	++	104
	4	++++	16		+			++	" 52	+++	104
	5	++++	16		+			−	−	+	104
	6	++++	16		+			+++	B. 63; E. 95	++++	

Group A. Conjunctival inoculations.

Group B. Sheath inoculations.

Group C. Controls.

? indicates result or condition uncertain; −, negative; +, positive where used with reference to lymph nodes, elsewhere indicates also slight or mild affection; ++, moderate affection; +++, well developed lesions or severe disease; ++++, marked lesions or very severe disease. B., bone; E., eye; M. M., mucous membranes; S., skin.

cutaneous chancre. The majority of them were situated in the region of the urinary meatus. In three instances the lesions measured as much as 6.0 to 8.0 mm. in their greatest diameter; they were clearly defined, well indurated, and showed some tendency to superficial necrosis and ulceration (Figs. 2, 5, and 6). The lesions in other animals were either extremely small, as in Fig. 7, or were rather ill defined processes of infiltration, and with the exception of the three animals in which the lesions were most characteristic, the conditions present might have been overlooked, had attention not been focussed on the genital mucosa, or they might have been mistaken for *cuniculi* lesions, had there been no other evidence of a syphilitic infection.

In like manner, from the same number of eye inoculations there were also seven instances in which definite lesions developed at the site of inoculation. In two animals the lesions were large, clearly defined granulomas. One of these involved the conjunctiva, episcleral tissues, and cornea and was associated with an erosion on the contiguous surface of the upper lid (Fig. 8), while the other was confined to the lower lid (Fig. 1). All other lesions were of the type of small indurated papules, most of which were on the margins of the lids, as in Fig. 9, suggesting that infection had taken place by entrance of spirochetes into the ducts of palpebral or sebaceous glands.

Of the three animals inoculated with the strain of low virulence, there was only one that developed a characteristic local affection, and even in this instance the lesions were very slight. This feature of the results serves to indicate that in experiments of this kind, the virulence of the organism used operates in precisely the same way as with other forms of inoculation.

Among the fifteen animals used in the three experiments, there were only eight in which a diagnosis of syphilitic infection probably could have been made on the basis of the lesions at the site of inoculation; three showed no definite lesions, and four showed slight lesions of indefinite character. Nevertheless, all of the animals presented other evidences of syphilitic infection. An inguinal adenitis was present in all of those inoculated on the genitalia, and nearly all of the animals showed a definite enlargement and induration of the popliteal lymph nodes. In addition, there were lesions in the testicles and scrotum.

The disease as a whole differed materially, however, from that in the control animals or from the disease ordinarily observed after mechanical inoculations of the testicles or skin. To begin with, the lesions at the site of inoculation were relatively small and ill defined; the incubation period was long, and in several instances definite lesions appeared at some remote point, such as the scrotum or testicles, before any lesion could be recognized at the site of inoculation or where no definite primary lesion occurred.

Moreover, the metastatic lesions in the testicles and scrotum were, like those at the site of inoculation, extremely slight. The scrotal lesions were all minute indurated papules (Fig. 3) or small plaques of infiltration, while those in the testicles assumed the form of small nodules or slight diffuse enlargements and infiltrations followed by atrophic changes. In only one instance (Table II, Group A, No. 3) was there an extensive orchitis and this did not develop until 7 months after inoculation.

The most notable features of the infection produced by this method of inoculation, however, were the absence of recognizable lesions in distant parts of the body other than those in the testicle and scrotum, and the extremely mild course of the disease throughout the prescribed period of observation.

The deviation from the usual course of events is most clearly brought out in the first experiment (Table I), where control animals were inoculated intracutaneously with the same dose of the emulsion as was used for the mucous membrane inoculations. In this instance, four of the six controls developed generalized lesions; in two instances there was an extensive involvement and the course of the disease was very severe. In fact, one animal was never able to overcome his infection and still showed extensive lesions at the time of death, 12 months after inoculation. There was another animal in which the disease was moderately severe, but the remaining three showed conditions that were comparable to the most pronounced infections in the experimental group.

In this connection, the fact should be pointed out that the results obtained in the control animals of this series showed that the virus used was highly active, and that other conditions that influence the course of syphilitic infections were especially favorable, as indicated by both the incidence and severity of generalized lesions.

A similar situation is brought out in the second experiment (Table II). Here, the purpose of the control was primarily that of a gauge of the activity of the virus and of the prevailing course of the disease, as indicated by a comparison with numerous experiments in which this method of inoculation had been used. There again, the results obtained in the control animals showed that the disease produced was more severe than usual. The incidence of generalized lesions was considerably increased and the involvement more extensive.

In this experiment, there were two possible cases of generalized disease among the animals inoculated on the mucous membranes, but in both the lesions were very slight. One animal developed a single papule on the sheath (autoinoculation ?) while, some 7 months after inoculation, the other developed a diffuse infiltration on the penis, which was indistinguishable from a *cuniculi* lesion.

Nothing can be said as to the generalized disease in the third experiment since this strain rarely produced generalized lesions by any method of inoculation, and those that occurred were always relatively slight.

DISCUSSION AND CONCLUSIONS.

The experiments reported show that under favorable conditions *Treponema pallidum* is capable of gaining entrance to the body of a normal rabbit when brought in contact with apparently normal mucous membranes. It is further shown that no recognizable lesion may occur at the portal of entry, or else it may be so slight as to escape detection; in any case, the lesion produced is apt to be small and ill defined as compared with primary lesions in the skin or testicles. Moreover, these lesions develop slowly and may not appear until some time after the development of lesions in distant parts of the body. Finally, it is shown that where infection originates in the manner indicated, the clinical course of the disease is apt to be entirely different from that produced by the usual methods of inoculation. With possible rare exceptions, the disease is extremely mild and in many instances, the existence of an infection might be overlooked, unless the most careful observations are carried out.

The explanation of these conditions is somewhat uncertain, but in general, the course of events is in line with what is known to occur

when infection takes place under conditions that are favorable to the host or are unfavorable to the spirochetes. In the first place, the failure to develop a typical chancre and the inconspicuous nature of the lesion at the portal of entry, as well as the prolonged incubation period, may be accounted for by the absence of any localizing influence, such as exists with other methods of inoculation, or in instances where there is a superficial trauma which favors both entrance and localization of spirochetes.

In like manner, the atypical course of disease may be attributable to the entrance of only a few organisms, or to lowering of the vitality of the spirochetes before they finally reach points that are favorable to their growth and multiplication. At all events, infection develops slowly, and wherever spirochetes become localized and begin to multiply, they are almost immediately suppressed, and for several months at least, they are unable to make sufficient headway to produce more than the most insignificant lesions in those parts of the body where the most pronounced lesions usually occur.

Unfortunately, the work had to be discontinued at this point and we were never able to complete the experiments that had been planned. But, the subsequent course of events may be predicted with a high degree of probability on the basis of the general principles of the interaction between *Treponema pallidum* and the animal host (5, 6). From what has been observed in many other experiments, it seems safe to assume that since no lesions of any consequence were produced during the first few months, the resistance or immunity developed was correspondingly slight and served mainly to curb the activity of the spirochetes in certain locations, but was not sufficient to suppress the infection or to prevent the occurrence of lesions in tissues that had not become involved. If this is true, as is the case with infections produced by other methods of inoculation, it would be expected that the infection would remain active longer than usual, and that from time to time new lesions would appear, and eventually the disease might assume relatively severe proportions with a tendency to the occurrence of lesions in parts that ordinarily would be protected by a vigorous reaction during the early stages of the infection. This did happen in the one animal of the series that was held beyond the allotted period of observation (Table II, Group A, No. 3).

The peculiar course of the infection observed in these animals appears to be closely analogous to the conditions that exist in those patients in whom syphilis pursues a mild or asymptomatic course during the early stages of the infection, and who eventually come under observation on account of some visceral affection or involvement of the central nervous system. As has been said, this course of events might be attributable, on the one hand, to an unusually high resistance on the part of individual patients, or to infection with an organism of low virulence, on the other. It is clear, however, that the same conditions may arise from still another cause; namely, the circumstances under which the infection is contracted. Just as the constitutional resistance of the patient or the virulence of the spirochete may affect the course of disease in accordance with well established principles of biological reaction, so also, such factors as the portal of entry, the mode of entrance, and the viability of the organism at the time of entrance into the body, may operate in such a manner as to increase or diminish the severity of the disease, or to change the clinical course of the disease in accordance with the same general principles of biological reaction.

It is believed, therefore, that the facts brought out in these experiments throw some light on the origin of obscure cases of syphilitic infection. Moreover, they are of equal importance in any consideration of conditions that are necessary for the production of an infection, or of factors that determine the clinical characteristics of the disease.

SUMMARY.

Experiments were carried out with three strains of *Treponema pallidum* to determine whether infection could be produced by applying an emulsion, rich in spirochetes, to normal mucous membranes of rabbits and whether an infection produced in this manner differed in any respect from one produced by other methods of inoculation.

It was found that a simple instillation of a spirochete emulsion into the conjunctival sac or the sheath was all that was necessary to obtain an infection. Still, the infection produced in this manner differed from that produced by intracutaneous or testicular inocula-

tions in that it tended to pursue a mild or asymptomatic course and frequently without the development of a characteristic chancre.

It is pointed out that these experiments may have a bearing on problems pertaining to obscure and atypical cases of human syphilis as well as the more immediate question of the infectivity of *Treponema pallidum*.

BIBLIOGRAPHY.

1. Fordyce, J. A., *Am. J. Med. Sc.*, 1915, cxlix, 781.
2. Reasoner, M. A., *Am. J. Syph.*, 1917, i, 478.
3. Chesney, A. M., *J. Exp. Med.*, 1923, xxxviii, 627.
4. Brown, W. H., and Pearce, L., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 200.
5. Brown, W. H., and Pearce, L., *J. Am. Med. Assn.*, 1921, lxxvii, 1619.
6. Brown, W. H., and Pearce, L., *New York State J. Med.*, 1923, xxiii, 376.

EXPLANATION OF PLATES.

Figs. 1 to 4 are reproductions of photographs not retouched; Figs. 5 to 9 are reproductions of colored drawings made directly from the objects represented. All objects are represented at their natural size. Statements of time are from the date of inoculation.

PLATE 33.

FIG. 1. 84 days. Chancre of the lower lid. (Table I, Group A, No. 1.)

FIG. 2. 49 days. Chancre of penis. Same animal as Fig. 1.

FIG. 3. 54 days. Inguinal adenitis with multiple papular lesions in both scrota. Same animal as in Figs. 1 and 2.

FIG. 4. 10 days. Inguinal adenitis in an animal inoculated with a strain of low virulence. There was no lesion at the site of inoculation.

PLATE 34.

FIG. 5. 64 days. A later stage of the lesion in Fig. 2, showing a clearly defined nodule at one side of the meatus.

FIG. 6. 82 days. A meatal chancre. (Table II, Group B, No. 3.)

FIG. 7. 54 days. A minute but clearly defined chancre on the penis. (Table II, Group B, No. 2.)

FIG. 8. 64 days. Primary granuloma of the eye. (Table I, Group A, No. 3.)

FIG. 9. 67 days. Multiple papular lesions on the margins of the lids. (Table II, Group A, No. 3.)

(Brown and Pearce: *Treponema pallidum*.)

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FIG. 5.

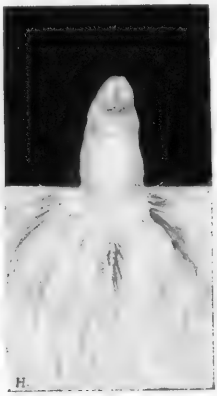

FIG. 6.

FIG. 7.

FIG. 8.

FIG. 9.

(Brown and Pearce: *Treponema pallidum.*)

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THE FATE OF ANTIGEN (PROTEIN) IN AN ANIMAL IMMUNIZED AGAINST IT.

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The purpose of the investigation has been to determine how the immune animal protects itself from the substance (antigen) against which it is immunized. Certain proteins have been selected as antigens because they are readily recognizable by specific precipitation. By means of this reaction their absorption from the site of injection and distribution throughout the body can be followed. Since the serum reactions of animals treated with antigens of bacterial origin are in considerable part similar to those produced by foreign proteins, observations made with these substances may point the way to a better understanding of bacterial immunity.

Protein substances such as horse serum and egg white injected into a normal animal readily pass from the site of injection into the circulating blood and are demonstrable in the serum during a period in most instances lasting from 7 to 9 days after injection. Egg white passes through the kidneys and may be demonstrated in the urine by means of the precipitin test. In a preceding study[1] I have shown that with the progress of immunization these antigens injected into the subcutaneous tissue or into the peritoneal cavity enter the circulating blood with decreasing readiness. In well immunized animals the substance fails to enter the circulating blood and cannot be demonstrated in the serum even though tests are made by the usual method of specific precipitation within the first few hours after injection. By injecting massive doses the capacity of the immunized animal to prevent entrance of the antigen into the blood may be over-

[1] Opie, E. L., *J. Immunol.*, 1923, viii, 55.

come but in this instance the antigen persists in the serum a much shorter time in the immune than in the normal animal.

Doerr and Pick[2] injected horse serum (1 to 2 cc.) into the peritoneal cavity of guinea pigs and examined the blood for antigen by means of the precipitin test. They found that it appeared in the blood of "sensitized" guinea pigs much more quickly than in the blood of normal animals. The sensitized animals had been injected with 0.1 cc. of horse serum 2 weeks before the experiment. Their observations repeated with the same result by Smith and Cook have little significance in relation to the problem considered in this paper.[3]

The absorption of antigen from the site of intracutaneous injection has been studied in guinea pigs by Smith and Cook. They reach the conclusion that injected serum disappears very rapidly from the site of injection in immunized animals, less rapidly in sensitized, and least rapidly in normal animals. In one experiment with uncertain result they studied two sensitized, two immune, and one normal animal. In a second experiment two sensitized, two immune, and two normal animals were used. Four injections at intervals of 12 hours were made into each animal and the desensitizing action of the first injection was not taken into consideration. The observations which I shall describe do not agree with those of Smith and Cook.

With the failure of antigen to enter the circulating blood of the immunized animal[1] in view, experiments have been undertaken to determine if it is held at the site of injection. It is noteworthy that the local reaction following injection differs widely in the normal and immune animal. The well known observations of Arthus have shown that foreign protein such as horse serum injected into the skin of a normal rabbit is absorbed without evident inflammatory reaction, whereas injected into the skin of an animal which has received five, six, or seven injections of the same antigen at intervals of 5 or more days there is intense inflammation. Abundant inflammatory edema occurs in a well localized area and there is necrosis of the skin and underlying tissue about the point of introduction of antigen.

In the first series of experiments crystalline egg albumin was employed in preference to horse serum or egg white which are complex

[2] Doerr, R., and Pick, R., *Centr. Bakt., 1. Abt., Orig.*, 1912, lxii, 146.

[3] Smith, G. H., and Cook, M. W., *J. Immunol.*, 1916–17, ii, 421.

mixtures of a variety of proteins. This substance acts as a simple antigen. Former experiments have shown that it causes complete disappearance of precipitin when injected in sufficient quantity into an immune animal and in consequence reactions for antigen and antibody are not obtained with the same blood.[1]

Disappearance of Crystalline Egg Albumin from the Site of Injection in Normal Rabbits.

A constant quantity of crystalline egg albumin, namely 0.5 cc. of a 5 per cent solution has been injected by means of a fine needle into the dermis over the abdomen. For convenience all results have been recorded in terms of this 5 per cent solution which represents the concentration of antigen received by the animal. At various intervals after injection skin has been removed and extracted with salt solution. A weighed amount of skin has been treated with salt solution in volume ten times its weight. Extraction has been facilitated by reducing the skin to a fine pulp by scraping with a knife and in earlier experiments by grinding in a mortar. The extract has been cleared by centrifugalization and repeated filtration through several layers of filter paper. It is essential that cellular debris and fat be removed so that the solution is clear or opalescent. During the latter part of the investigation centrifugalization at high speed was found sufficient.

The extracts were tested with the sera of rabbits highly immunized against crystalline egg albumin. 1 cc. of the diluted extract was treated with 0.2 cc. of the immune serum. The method of estimating the concentration of antigen in the extract is illustrated below. The relation of weight of tissue to volume of water does not permit an exact estimate of the concentration of the antigen in the tissue but dividing by 10 gives a figure which is below that of the actual content and is comparable from one experiment to another. In later experiments with horse serum this error has been corrected.

Into the dermis of a normal rabbit (Rabbit 1)[4] was injected 0.5 cc. of a 5 per cent solution of crystalline egg albumin. After 24 hours the animal was killed

[4] Consecutive numbers were given to these animals after the experiments had been completed.

and skin weighing 1.38 gm. removed from the site of injection was used for the preparation of an extract by addition of 13.8 of salt solution. For control a similar extract was prepared from 1.13 gm. of skin removed from the opposite side of the abdomen. These extracts were tested for antigen with two sera from rabbits repeatedly injected with crystalline egg albumin.

1 cc. of dilution of extract.	Extract from site of injection tested with 0.2 cc. of serum.			Extract from normal skin tested with 0.2 cc. of serum.		
	From Rabbit 2.	From Rabbit 3.	From normal rabbit.	From Rabbit 2.	From Rabbit 3.	From normal rabbit.
1:2	+++	+++	0	0	0	0
1:5	++	++	0	0	0	0
1:10	+	+	0	0	0	0
1:20	+	+	0	0	0	0
1:50	0	+	0	0	0	0
1:100	0	0	0	0	0	0
1:200	0	0	0	0	0	0
1:500	0	0	0	0	0	0

The strength of the two sera used in this experiment is indicated by the following tests made in the usual manner.

1 cc. of dilution of 5 per cent crystalline egg albumin.	With 0.2 cc. of serum.	With 0.2 cc. of serum.
	From Rabbit 2.	From Rabbit 3.
1:100	+	+
1:1,000	+++	++++
1:10,000	++	+++
1:20,000	+	+
1:50,000	+	+
1:100,000	0	Trace.
1:1,000,000	0	0

Comparison between the tests for antigen in extracts from the site of injected crystalline egg albumin and the foregoing tests made with known dilutions of the antigen indicates that the antigen was present in the extract in concentration of 1/2,500 and in the tissue of approximately 1/250.

The blood serum of the animal drawn 24 hours after injection of the antigen gave a reaction for crystalline egg albumin in dilution of 1:5 representing a concentration of antigen within the serum of approximately 1/10,000.

An extract was prepared from 1.38 gm. of skin removed 2 days after the injection of 0.5 cc. of 5 per cent crystalline egg albumin (Rabbit 4). This extract was tested with a serum which formed a precipitate with the antigen diluted 1:100,000.

1 cc. of dilution of extract.	Extract from site of injection with 0.2 cc. of serum.	
	From Rabbit 2.	From normal rabbit.
1:2	++	0
1:5	++	0
1:10	+	0
1:20	+	0
1:50	0	0
1:100	0	0
1:200	0	0

Comparison with tests made by using known dilutions of the antigen indicates that the concentration of antigen in the extract was 1/500. Crystalline egg albumin was not found in the blood serum.

Tests for antigen were made with an extract prepared from skin, 3 by 2 cm., excised at the site of injection of 0.5 cc. of 5 per cent solution of crystalline egg albumin introduced 3 days before the animal (Rabbit 5) was killed. Similar tests were made with an extract of skin removed from the opposite side of the abdomen. Each extract was tested with two strong sera (with titer of 1:50,000) from rabbits (Rabbits 2 and 3) which had received crystalline egg albumin and normal serum was used as control. Tests with dilutions of the extract varying from 1:2 to 1:500 were uniformly negative. The blood serum of the rabbit 3 days after injection of crystalline egg albumin contained none of the antigen.

An extract prepared from 0.672 gm. of skin 3 days after the injection of 0.5 cc. of 5 per cent solution of crystalline egg albumin into a normal rabbit (Rabbit 6) was tested for the antigen by means of a strong serum with titer of 1:50,000 (from Rabbit 3, repeatedly injected with crystalline egg albumin) and the result was negative. The blood contained none of the antigen.

These experiments have shown that crystalline egg albumin injected into a normal animal disappears rapidly from the site of injection and at the end of 3 days is no longer demonstrable. The substance enters the blood but disappears very quickly and has not been found after the 1st day. In this respect crystalline egg albumin differs from horse serum which persists during at least 1 week. Crystalline egg albumin is readily demonstrated in the urine of normal rabbits during the first 24 hours after intradermic injection. Examination of the urine for horse serum under similar conditions has been uniformly negative.

Disappearance of Crystalline Egg Albumin from the Site of Injection in Immune Rabbits.

Rabbits were immunized against crystalline egg albumin by five or more injections of 0.5 cc. of a 5 per cent solution introduced into the subcutaneous tissue of the back. The test injection was made into the dermis of the abdominal wall and was in all instances followed by inflammatory swelling with necrosis (Arthus phenomenon).

Rabbit 7 which had been immunized against crystalline egg albumin was given an intradermic injection of 0.5 cc. of a 5 per cent solution of the substance. 24 hours later there were extensive swelling and necrosis of the skin. Extracts were prepared (*a*) from the necrotic dermis together with a narrow margin of living skin (1.587 gm. with 15.87 cc. of salt solution) and (*b*) from the edematous subcutaneous tissue below the site of injection (1.97 gm. with 19.7 cc. salt solution.) These extracts were tested for antigen by means of an immune serum.

1 cc. of dilution of extract.	Extract from necrotic skin at site of injection tested with 0.2 cc. of serum.		Extract of subcutaneous tissue at site of injection tested with serum.	
	From Rabbit 3.	From normal rabbit.	From Rabbit 3.	From normal rabbit.
1:2	+++	0	+++	0
1:5	++	0	++	0
1:10	++	0	+	0
1:20	+	0	+	0
1:50	+	0	Trace.	0
1:100	+	0	0	0
1:200	Trace.	0	0	0
1:500	0	0	0	0
1:1,000	0	0	0	0

The concentration of antigen estimated by comparison with the reactions produced by immune serum from Rabbit 3 with known dilution of crystalline egg white was approximately 1:20 in the necrotic skin at the site of injection and 1:80 in the underlying subcutaneous tissue. No antigen was found in the blood.

Crystalline egg albumin (0.5 cc.) was injected into the dermis of Rabbit 2 which had been many times injected with the antigen. The serum of this animal exhibited a precipitin reaction with the antigen solution diluted 1:100,000. An intense reaction followed the injection and there was edematous swelling in an area 7.1 by 5.8 cm. with extensive necrosis of the skin. Necrotic skin and underlying tissue (1.35 gm.) removed by operation under ether anesthesia were extracted with salt solution (13.5 cc.). Tests for antigen were as follows:

1 cc. of dilution of extract.	Extract from site of injection tested with 0.2 cc. of serum.		
	From Rabbit 8.	From Rabbit 2.	From normal rabbit.
1:2	+++	+++	0
1:5	+++	+++	0
1:10	++	++	0
1:20	+	+	0
1:50	+	+	0
1:100	+	+	0
1:200	Trace.	Trace.	0
1:500	0	0	0

Concentration of antigen in the tissue estimated by comparison with known dilutions of crystalline egg albumin and serum from Rabbit 8 is 1/50; tested with serum from Rabbit 2 the same figure is obtained.

Injection of 0.5 cc. of crystalline egg albumin into the dermis of Rabbit 3 which had received numerous injections of the antigen caused extensive swelling (6.2 by 8.4 cm.) with necrosis of the skin. An extract was made from the skin and underlying tissue (1.785 gm.) removed 3 days after injection. The results of tests for antigen were as follows:

1 cc. of dilution of extract.	Extract from skin at site of injection tested with 0.2 cc. of serum.		
	From Rabbit 2.	From Rabbit 8.	From normal rabbit.
1:2	+++	+++	0
1:5	++	++	0
1:10	+	+	0
1:20	+	Trace.	0
1:50	Trace.	"	0
1:100	0	0	0
1:200	0	0	0
1:500	0	0	0

Estimated with the same result by means of sera from Rabbit 2 and from Rabbit 8 the concentration of antigen at the site of injection after 3 days is 1/250. There was no antigen in the serum of Rabbit 3 and the precipitin titer 3 days after injection was 1:100,000.

Rabbit 7 which had been repeatedly injected with crystalline egg albumin received in the dermis 0.5 cc. of the solution. Extensive swelling over an area 8.6 by 7 cm. occurred at the site of injection and there was a focus of necrosis at its center. Swelling was still present after 4 days, when the site of injection was removed and used (0.97 gm.) for the preparation of an extract. The result of tests for antigen made with this extract were as follows:

1 cc. of dilution of extract.	Extract from site of injection tested with 0.2 cc. of serum.	
	From Rabbit 3.	From normal rabbit.
1:2	+	0
1:5	Trace.	0
1:10	"	0
1:20	0	0
1:50	0	0
1:100	0	0
1:200	0	0
1:500	0	0

Concentration of antigen estimated by comparison of above tests with the effect of immune serum (from Rabbit 3) upon known dilutions of crystalline egg albumin indicated that the concentration of antigen in the tissue at the site of injection was approximately 1/750.

Into the dermis of Rabbit 2 which had received many injections of crystalline egg albumin was injected 0.5 cc. of a 5 per cent solution of the antigen. Extensive swelling occurred in an area 7 by 6 cm. and there was necrosis of skin in a triangular area 2.5 by 4.5 cm. Swelling persisted little diminished during 5 days when the animal was killed and tissue was removed for examination. Separation of the necrotic from the living skin had begun. The surrounding swollen tissue when sectioned was found to be edematous but firm grayish white and in places indurated. Extracts were made (*a*) from the necrotic skin separated from the adjacent tissue (2.65 gm. with 26.5 cc. of salt solution) and (*b*) from the edematous and indurated tissue nearby (4.27 gm. with 42.7 cc. of salt solution). Tests for antigen were as follows:

1 cc. of dilution of extract.	Extract of necrotic skin tested with serum.		Extract of indurated tissue at site of injection tested with serum.	
	From Rabbit 2.	From normal rabbit.	From Rabbit 2.	From normal rabbit.
1:2	++	0	0	0
1:5	++	0	0	0
1:10	+	0	0	0
1:20	+	0	0	0
1:50	0	0	0	0
1:100	0	0	0	0
1:200	0	0	0	0

Estimated concentration of antigen in the necrotic skin was 1/200; there is no antigen in the living tissue.

The figures representing the concentration of antigen in the tissue are comparable throughout. Living tissue containing a small focus of necrosis removed from immune Rabbit 7, 24 hours after injection was tested for antigen and the result is included in the table below. In immune Rabbit 2, 5 days after injection antigen was found to have persisted in the necrotic tissue which was in process of sequestration but none was found in the surrounding, living tissue. The rate of disappearance of crystalline egg albumin from the site of injection in normal and immune animals as indicated by the foregoing experiments is shown by the following table.

Rabbit No.	Normal.		Rabbit No.	Immune.	
	Days after injection.	Concentration of antigen in tissue.		Days after injection.	Concentration of antigen in tissue.
1	1	1/250	7	1	1/20 to 1/80
4	2	1/500	2	2	1/50
5	3	0	3	3	1/250
3	3	0	7	4	1/750
			2	5	0

Crystalline egg albumin introduced into the skin of normal animals disappears very rapidly from the site of injection and is no longer demonstrable after 3 days. In immune animals it is held in greater concentration at the site of injection, diminishes slowly, and has not disappeared until the 5th day. My former observations have shown that crystalline egg albumin injected into a normal rabbit enters the blood and is demonstrable in the blood serum; furthermore, it is excreted in the urine. In an immune animal, on the contrary, crystalline egg albumin has failed to enter the blood and in the few instances in which the urine has been tested the result has been negative. The immunized animal has acquired the ability to fix this relatively diffusible substance at the site of introduction so that it is not disseminated by the circulating blood.

Disappearance of Horse Serum from the Site of Injection in Normal and in Immune Animals.

Since horse serum has been used in many investigations concerning the relation of antigen to antibodies it has been considered desirable to repeat with this substance the foregoing experiments. Horse serum is evidently less diffusible than crystalline egg albumin and in the tests which I have made, 0.5 to 1 cc. of horse serum being injected into the skin, has failed to enter the urine either in the normal or in the immune animal. Like crystalline egg albumin it enters the blood of the normal animal but fails to make its appearance in the blood serum of immunized animals.

In each of the following experiments 0.5 cc. of horse serum was injected into the dermis over the abdomen of a normal rabbit. At the end of 1, 2, or 3 days after the injection the animal was killed by bleeding from the femoral artery under ether. The site of injection was not marked by any swelling but on section after 24 hours it contained a slight excess of fluid which had wholly disappeared after 2 days. Skin from the site of injection was scraped to a fine pulp with a knife and extracted with a measured quantity of salt solution as in the foregoing experiments. For control a piece of skin from the opposite side of the abdomen was subjected to the same treatment. The extract was tested for the presence of horse serum by adding 0.2 cc. of anti-horse serum from rabbit to dilutions of the extract.

Rabbit 9 was killed 24 hours after injection of horse serum and a weighed quantity of skin from the site of injection (3.95 gm.) was extracted with a volume of salt solution ten times this weight; skin from the opposite side of the abdomen (3.55 gm.) was used as control.

1 cc. of dilution of extract.	Extract of skin from site of injection tested with		Extract of skin from opposite side of abdomen tested with	
	0.2 cc. of anti-horse serum from Rabbit 10.	0.2 cc. of normal serum.	0.2 cc. of anti-horse serum from Rabbit 10.	0.2 cc. of normal serum.
1:10	++++	0	0	0
1:50	+++	0	0	0
1:100	++	0	0	0
1:200	+	0	0	0
1:500	0	0	0	0

Comparison of these reactions with those obtained by adding immune serum to known dilutions of horse serum showed that the concentration of horse serum remaining in the tissue 24 hours after injection was 1/15.

In the next experiment Rabbit 11 was killed after 2 days and extracts were prepared as before from the skin at the site of injection (0.515 gm.) and from the opposite side of the abdomen (0.365 gm.).

1 cc. of dilution of extract.	Extract of skin at site of injection tested with.		Extract of skin from opposite side of abdomen tested with.	
	0.2 cc. of serum from Rabbit 10.	0.2 cc. of normal serum.	0.2 cc. of serum from Rabbit 10.	0.2 cc. of normal serum.
1:5	+++	0	0	0
1:10	++	0	0	0
1:50	+	0	0	0
1:100	Trace.	0	0	0
1:200	0	0	0	0
1:500	0	0	0	0

Concentration of the horse serum remaining in the skin at the site of injection was approximately 1/100. Precipitin tests made with the blood serum of this animal showed that horse serum was present and comparison with known dilutions of horse serum showed that its concentration was 1/500.

A similar experiment (Rabbit 12) performed with skin removed 3 days after the injection of horse serum showed that the antigen had so completely disappeared from the site of injection that it was no longer demonstrable by strong anti-horse serum from the rabbit.

In the following experiments horse serum (0.5 cc.) was injected into the dermis over the abdomen of rabbits which had been immunized by subcutaneous injections of horse serum (1 cc.) repeated at intervals of 5 days. An intense inflammatory reaction (Arthus phenomenon) occurred at the site of injection; there was abundant edema in a circumscribed area, and necrosis of the skin and underlying subcutaneous tissue occurred in the center of the swollen area at the point of introduction of the antigen.

2 days after the introduction of 0.5 cc. of horse serum into the dermis of Rabbit 13, which had received eight injections of the antigen, there was swelling in an area 6 by 3.5 cm. raised about 1 cm. above the general level of the surface and near the center of this area there was a spot of purplish black necrosis 1.1 cm. across. A piece of skin 3 by 2 cm. containing the central spot of necrosis weighed 2.36 gm. and was extracted with 23.6 cc. of salt solution. The remainder of the edematous area weighing 17.72 gm. contained red serous fluid which was readily pressed from

the tissue by scraping with a knife; 2.65 gm. of this fluid were mixed with 2.65 cc. of salt solution.

1 cc. of dilution of extract.	Extract from skin showing necrosis with.		Serous effusion from edematous skin with.	
	0.2 cc. of serum from Rabbit 10.	0.2 cc. of normal serum.	0.2 cc. of serum from Rabbit 10.	0.2 cc. of normal serum.
1:5	++++	0	++++	0
1:10	++++	0	++++	0
1:50	++	0		
1:100	+	0	+++	0
1:200	+	0	++	0
1:500	Trace.	0	+	0
1:1,000	0	0		

Comparison between these reactions and those of known quantities of horse serum indicates that the concentration of the antigen in the skin with necrosis was 1/20 whereas that in the serum from the edematous tissue was approximately the same.

Experiments in which extracts were prepared from the inflammatory tissue at the site of injection, 3 (Rabbit 14), 4 (Rabbit 15), and 6 days (Rabbit 16, tissue removed by operation) after horse serum had been introduced into the dermis of well immunized animals showed that the antigen was no longer demonstrable by the precipitin test.

Concentration of antigen at the site of injection 1 and 2 days after introduction of 0.5 cc. of horse serum into a normal animal was, respectively, 1/15 and 1/100, whereas 2 days after injection into an immune animal the concentration was 1/20 indicating that the absorption proceeded more slowly in the latter. Nevertheless both in the immune and in the normal animal antigen has disappeared after 3 days.

The concentration of antigen in the excised skin does not furnish an accurate measure of the quantity of antigen remaining at the site of injection, for in the normal animal there is no evident reaction, whereas in the immune animal there is an inflamed and edematous focus which excised 1 or 2 days after injection weighs approximately 20 gm. The antigen in the immune animal has been diluted by a large accumulation of edematous fluid. Concentration of antigen in the fluids of the tissue would furnish a means of estimating its total quantity provided the volume of fluid in the tissue were known.

Tissue removed has been cut into two parts; one part has been extracted with the purpose of determining the concentration of antigen, and the other has been dried at 100°C. to a constant weight to determine the ratio of water to solid material. A simple calculation will determine the total volume of water in the excised tissue of which the weight is known and from the concentration of antigen as determined by the precipitation test the quantity of antigen in the tissue is deduced. In the following experiment the quantity of antigen remaining in the skin 24 hours after injection into a normal animal is compared with that present after the same interval in an immune animal.

At the end of 24 hours after injection of 0.5 cc. of horse serum into the dermis of normal Rabbit 17 precipitation tests showed that the antigen had entered the blood serum and was present in concentration of 1/500. (*a*) To determine the quantity still present at the site of injection a large oval patch of skin 7.5 by 4 cm. was removed. (*b*) To determine the extent of diffusion within the skin a zone of skin surrounding (*a*) and 1.5 cm. broad was removed; (*c*) a second zone surrounding (*b*) and with the same breadth was preserved for examination. In each instance part of the tissue removed was extracted with normal salt solution and examined for antigen by the precipitin test; part was desiccated at 100°C. to determine the water content.

	Weight of tissue.	Water content.	Concentration of horse serum in water of tissue.	Estimated amount of horse serum present.
	gm.	*cc.*		*cc.*
(*a*).....	3.8	2.4	1/20	0.12
(*b*).....	5.245	3.375	53/500	0.03
(*c*).....	8.69	4.6	65/1,500	0.02
				0.17

Approximately one-third of the injected protein is recoverable at and about the site of injection. Wide diffusion of the horse serum has occurred within the skin so that it is present in small quantity in a zone 3 cm. from the point of introduction.

The same method was employed to determine the fate of protein injected into immune Rabbit 18. Its blood caused a precipitate with horse serum diluted 1:100,000. At the end of 24 hours following the intradermic injection of 0.5 cc. of

horse serum no antigen was demonstrable in the blood serum. There were extensive swelling and necrosis at the site of injection which was included in (*a*) an oval piece of skin measuring 13 by 5 cm. A zone of skin (*b*) 1.5 cm. across surrounding the inflamed area was excised for examination and extracts were prepared.

	Weight of tissue.	Water content.	Concentration of horse serum in water of tissue.	Estimated amount of horse serum present.
	gm.	*cc.*		*cc.*
(*a*)	24.795	20.253	1/44	0.46
(*b*)	9.54		0	0

The blood serum was tested for the presence of horse serum with negative result.

Tests made 24 hours after injection of the antigen give the most definite indication concerning the fate of antigen at the site of introduction for at a later period antigen disappears both in the normal and in the immune animal. In the normal animal only one-third of the antigen has been recoverable in a wide area of skin surrounding the point of injection and the extraction of contiguous zones of skin has shown that widespread diffusion has occurred within the skin itself. Furthermore the antigen has entered the blood and has been carried to all parts of the body. In the immune animal, on the contrary, almost all of the antigen has been accounted for at the site of injection, namely 0.459 cc. of 0.5 cc. injected. It has been limited to the focus of inflammation and has not been demonstrable in the surrounding zone of skin. None has been found in the blood.

The attempt was next made to extract and measure directly the entire amount of protein retained in the tissue at the site of injection. The excised skin was covered with distilled water and allowed to stand in the ice chest during 1 or 2 days; the tissue, now softened and distended by the water, was scraped to a pulp and again permitted to stand overnight. The mixture was centrifugalized and the fluid which separated was poured off and measured. The material which settled to the bottom was weighed with the tube, dried to a constant weight at 100°C., and again weighed to determine the amount of water. The concentration of horse serum in the centrifugalized fluid was determined by the precipitin test and the total quantity of fluid being known, the quantity of antigen in solution was determined.

An extract was prepared from the skin removed 24 hours after injection of 0.5 cc. of horse serum into the dermis of normal Rabbit 19. The skin excised from the site of injection (*a*) was oval measuring 18 by 10 cm. A zone of skin (*b*) surrounding this oval patch and 1 to 1.5 cm. broad was excised in order to determine how far the antigen had diffused in the tissue surrounding the point of introduction. The figures obtained were as follows:

	Weight of skin extracted.	Total fluid of extract.	Concentration of horse serum in extract.	Estimated quantity of horse serum.
	gm.	*cc.*		*cc.*
(*a*)	42.69	54.625	1/200	0.273
(*b*)	10.815	26.85	1/2,000	0.011
				0.284

Antigen had entered the blood and was present in concentration of 1/750.

The injected protein had not only entered the blood but had diffused so widely in the tissue that it was found more than 5 cm. from the point of introduction.

The same experiment was repeated with Rabbit 16 which was well immunized. Introduction of 0.5 cc. of horse serum was followed by extensive inflammatory edema with necrosis. The inflamed area was excised and extracted with distilled water. The following figures were obtained.

Weight of excised tissue.	Total fluid of extract.	Concentration of horse serum in extract.	Estimated quantity of horse serum.
gm.	*cc.*		*cc.*
17.07	44.886	1/100	0.449

The blood serum of the rabbit was examined for horse serum with negative result.

This experiment comparing normal and immune animals confirms the preceding experiment (Rabbits 17 and 18) for in the normal animal only half of the antigen has been recovered at the site of injection; it has diffused widely in the tissue and has entered the blood. In the immune animal almost all of the injected antigen is still present within the inflamed tissue at the site of its introduction and none is demonstrable in the blood.

The experiments show that crystalline egg albumin injected into a normal animal leaves the site of injection with much greater rapidity than horse serum. The substance appears to pass very readily not only through vascular endothelium but through the kidney as well so that it appears in the urine and is eliminated from the blood. The immune animal has acquired the ability to fix this readily diffusible substance at the site of its introduction. The possibility that the antigen enters the blood in very small quantity cannot be excluded but none has been demonstrable. The substances which have been employed are relatively though by no means absolutely harmless so that the protection afforded by local fixation is not obvious. Nevertheless it is evident that injurious action of the antigen in the immune animal will be limited almost wholly to the site of its entry.

It is noteworthy that the substances employed produce little recognizable local change when injected into a normal animal, but in the early stages of immunity edematous swelling, accumulation of polynuclear leucocytes, and other manifestations of acute inflammation follow entry of the foreign protein. In a well immunized animal, necrosis of tissue shows the severity of the injury. These changes constitute the phenomenon of Arthus which is incorrectly designated skin hypersensitiveness.

The data available do not explain how the protein injected into the immune animal causes inflammatory exudation and destruction of tissue. Contact of horse serum such as occurs at the site of injection in the normal animal during the first 24 hours is insufficient to produce these changes even though the concentration of antigen is as great as that in the immune animal. Specific precipitation of protein doubtless occurs in the immune animal and has a part in the local fixation of antigen and in the production of inflammation. The occurrence of the inhibition zone in the precipitation reaction indicates that excess of antigen will for a time prevent formation of precipitate at the point of injection where the concentration of antigen is highest (*e.g.* 1/20 to 1/50) but with diffusion of antigen into adjacent tissue a dilution would be reached favorable to the formation of precipitate. Previous experiments have shown that precipitation occurs when antigen and precipitin come together in

the blood serum and there is no reason to doubt that specific precipitation occurs within the fluids of the tissue.

Injection of washed precipitate formed by the action of precipitin upon horse serum has shown that the precipitate is strongly chemotactic for polynuclear leucocytes. It is probable that the destruction of injected antigen is brought about by intracellular digestion. With immunization the animal acquires the ability to fix antigen at the site of introduction thus preventing its dissemination throughout the body. Local fixation of antigen is associated with acute inflammation and even with necrosis of tissue, but destruction of the antigen occurs in the inflamed tissue at the site of fixation. The apparent susceptibility of the protected animal to local injury of tissue is a paradox explained by changes which serve to protect the organism as a whole.

CONCLUSIONS.

When proteins such as horse serum or crystalline egg albumin which have been selected because they produce the phenomena of immunity are introduced into a normal animal they diffuse widely in the tissue, enter the blood stream, and are disseminated throughout the body.

The same substances introduced into an immune animal are fixed at the site of entry and are not found in the blood.

When protein is injected into the skin of an immune animal acute inflammation (Arthus phenomenon) occurs at the site of injection and brings about destruction of the foreign substance.

STUDIES ON THE RESPIRATORY MECHANISM IN LOBAR PNEUMONIA.

A Study of Lung Volume in Relation to the Clinical Course of the Disease.

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Plates 35 and 36.

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INTRODUCTION.

The present study has been undertaken in order to obtain data concerning the relationships in pneumonia between the extent and progress of pulmonary involvement, the occurrence of dyspnea and cyanosis, and the changes in lung volume.

In a disease such as lobar pneumonia, it is impossible to measure the vital capacity or the total lung capacity. The patients are too ill to be permitted to make the effort required for maximal inspiration and expiration. Factors, such as psychic state, pleuritic pain, and general muscular weakness would furthermore introduce elements which might easily vitiate the accuracy of measurements involving forced breathing. For this reason it was determined not to attempt to make observations on total lung capacity, vital capacity, or residual air. The mid-capacity (Panum (1); Bohr (2)), we know, forms a fairly constant fraction of the total lung capacity and varies with it. We have chosen the volume of air remaining in the lungs in the resting expiratory position. For this lung volume we have preferred the term given by Lundsgaard (3), *functional residual air*. This has been studied by Siebeck (4) and Krogh (5) and is sometimes called the mid-capacity. As its name implies, it represents that volume of air which at the end of a normal expiration remains in the lungs and air passages. It must, therefore, express in a quantitative sense, the state of pulmonary distention, and indirectly the surface area of pulmonary epithelium through which diffusion occurs.

Anyone who has made observations on the respiratory movements in man will have noticed that the expiratory pause occurs at a more constant point than the inspiratory. This fact has been emphasized by Krogh (6), Benedict and Collins (7), and Hendry, Carpenter, and Emmes (8). In a graphic tracing of respiration, such as is made in the course of metabolism studies with a Benedict apparatus (Roth (9)), it will be seen that a straight line can be drawn more readily through the expiratory than through the inspiratory points. This has determined us in selecting the expiratory rather than the inspiratory position for measurement.

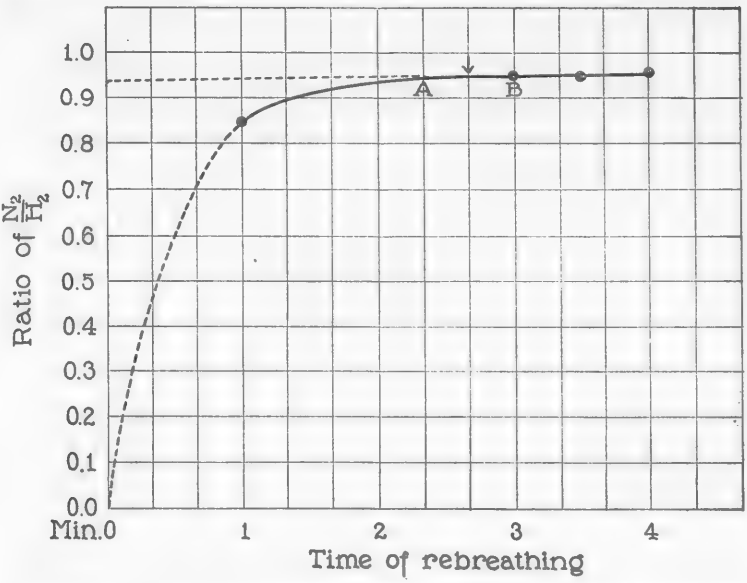
The functional residual air in a given normal individual is constant within small fluctuations under constant conditions of rest and bodily position. The average deviation for a series of thirteen individuals amounted to but ± 40 cc. in an average lung volume of 2.37 liters. This afforded us our normal criteria. Changes of 100 cc. or more in lung volume measurements are to be regarded as related to the disease process and not as a consequence of normal functional variations or analytical errors.

Methods and Material.

The patient to be investigated was wheeled to the laboratory in his bed. The apparatus was so screened that little of it was exposed to his view. After some preliminary investigation it was found that the ordinary wide flanged rubber mouthpiece was preferrable to the mask for pneumonia patients. During the period of dyspnea most patients breathe through their mouths and so do not object to having the nares closed, if it be done gently and gradually. The pressure on the face necessary to make a mask fit snugly produces a sense of suffocation in most patients with pneumonia. We have found it better to have a nurse hold the nose closed than to use a mechanical nose clip. It gives the patient a sense of confidence and is more comfortable. A little vaseline placed in the nares considerably lessens the discomfort of pinching the nose.

Method for Measuring Functional Residual Air.—For measuring functional residual air the method of Van Slyke and Binger (10) was used, with modifications. These consisted of certain technical changes outlined below: (*a*) a graphic recording device on the mixing spirometer; (*b*) substitution of all metal parts in the circulation apparatus for the purpose of rendering this more surely proof against leaks and more readily sterilizable; (*c*) substitution of flutter valves (Sadd valves) in place of Douglas flap valves; (*d*) substitution of a two-way aluminum stopcock with minimum dead space for the five-way cock previously used; and (*e*) collection of gas samples in previously evacuated mercury sampling tubes.

This latter insured almost instantaneous collection of samples from the mixing spirometer at the time desired. Because of the relatively slow mixing of spirometer gas with lung air in normal and shallow breathing, it was necessary to collect the samples at somewhat different intervals than was practised in measuring the



TEXT-FIG. 1. Typical mixture curve from which the lung volume is calculated. At the first point on this curve, mixture is obviously not complete. The last three points fall on a straight line, which indicates complete mixture. The upward inclination of this line is discussed in the text.

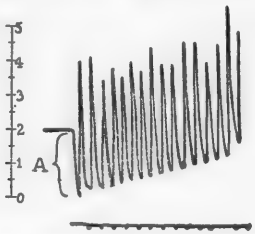
Lung volume is calculated from the point indicated by the arrow half way between *A* and *B*; *viz.*,

$$0.95 \times \frac{1}{79.1} \times 2 \text{ (liters of hydrogen)} = 2.40 \text{ liters (uncorrected lung volume).}$$

The part of the curve indicated by the broken line is imaginary.

residual air (*cf.* Van Slyle and Binger (10) and Binger (11)), in which case the first deep inspiration after obtaining vital capacity accomplished most of the gas mixture. The intervals of sampling and the type of mixing curve established from the $\frac{N_2}{H_2}$ ratio of a typical case are shown in Text-fig. 1.

A sample was taken at a point, usually within the 1st minute of breathing, before mixture was complete, and three successive samples at ½ minute intervals were drawn after mixture was known to be complete between the 3rd and 4th minutes of breathing. The $\frac{N_2}{H_2}$ ratios obtained from these three samples almost invariably fell on a straight line, inclining slightly upward. This upward inclination was discussed by Van Slyke and Binger (10) and can best be interpreted as due to the gradual absorption of H_2 and elimination of N_2. It cannot be due to incomplete mixture because a mixture curve could not be straight and would necessarily have diminishing increments. By drawing a curve from the zero point



TEXT-FIG. 2. Tracing from the mixing spirometer demonstrating the method of correction for position. In this figure and in the succeeding one, a down stroke of the pen indicates expiration. The stop-cock was turned at the end of the horizontal portion of the tracing. At this point the volume of air in the subject's lungs exceeded his true functional residual air by a volume represented by the distance *A* on the tracing. The factor for the spirometer is represented by an excursion of the bell of 4.82 cm. for every liter. $A = 2$ cm. The correction to be subtracted from the observed lung volume in this case amounts therefore to $\frac{2}{4.82 \text{ cm.}}$, or 415 cc. The scale represents centimeters reduced proportionately to the tracing.

through the point of incomplete mixture to the line representing complete mixture, a small region is delimited in which mixture must be just complete. The mean between points *A* and *B* (Text-fig. 1) is taken to give the $\frac{N_2}{H_2}$ ratio from which the lung volume is calculated. In all lung volume determinations in this study we used 2 liters of H_2 and 2 liters of O_2 in the mixing spirometer. A correction due to air content in the spirometer amounting to 100 cc. was subtracted from each lung volume determination. This was discussed by Van Slyke and Binger (10). Lung volumes were not reduced to standard temperature and pressure but are given under observed conditions.

A further correction for position was necessary in each determination to assure obtaining the true expiratory position or functional residual air. This was accomplished by the simple procedure of turning the stop-cock, through which the patient

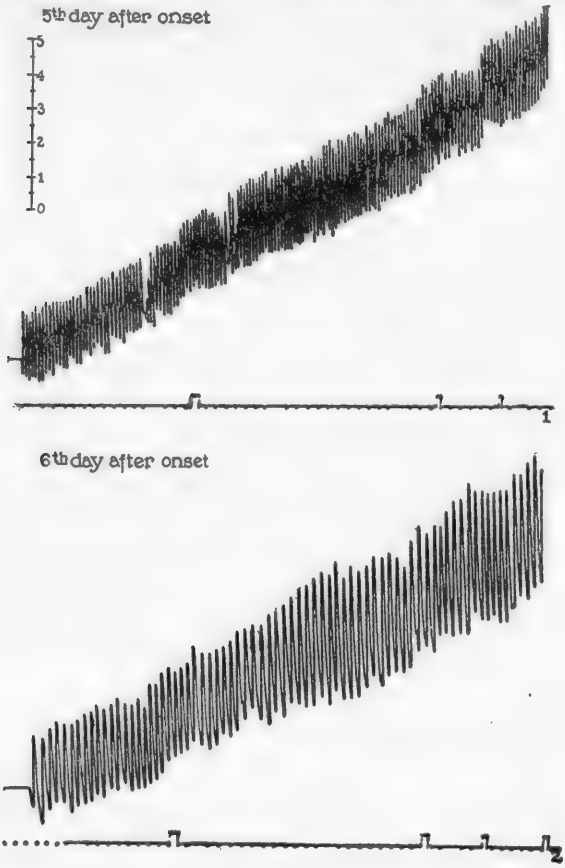
had been breathing room air, while he was in the expiratory phase of respiration. His first breath after connection with the mixing spirometer was therefore into it rather than from it. And the volume of this first breath represented the excess of air in the patient's lungs above that of the resting expiratory position. This is shown in Text-fig. 2. By this procedure the chief source of error in lung volume determinations is obviated.

Text-fig. 3 shows the type of breathing recorded by the mixing spirometer at various stages of the disease in one patient. The slight increase in amplitude at the end of each tracing is due to incomplete scrubbing of air, which permitted slight CO_2 accumulation. The slope in the curves gives an indication of the rate of O_2 consumption. Note the greater respiratory rate and smaller volume of tidal air at the beginning than at the end of disease, and the greater rate of O_2 consumption.

The Instrumental Recording of Respiratory Rate and Depth.—To obtain a quantitative graphic tracing of the pulmonary ventilation in the human subject which will approximate his normal, *i.e.* unrecorded, breathing is very difficult. Even with every precaution for freedom from mechanical resistance, complete CO_2 removal, and oxygen replenishment, many normal untrained subjects will alter their type of breathing when connected with a spirometer. This alteration is still more pronounced in the pneumonia patient. We have observed the breathing of these patients to be consistently slower, and probably deeper, when it is measured graphically by spirometry than when it is counted at the bedside on the ward. For this reason, in spite of a mass of data on normal subjects and peumonia patients, we have depended on the counted respiratory rate as evidence of pathological breathing rather than on the measurements made from spirometer tracings. For it is obvious that if the respiratory rate is altered, the minute volume of pulmonary ventilation will likewise be altered. It should be said, however, that in most of the pneumonia patients the characteristic rapid and shallow breathing with increase of minute volume was observed as compared with normal subjects or with the same patient when convalescent. Text-fig. 3 exhibits the progressive changes which are seen in type of breathing during convalescence from the disease. A pause at the end of expiration is usually seen in normal tracings but is missing when breathing is rapid.

The material for study comprised twelve cases of lobar pneumonia of various types. The results from the first two of the series had to be discarded because of errors of technique. Of the remaining ten, seven were men and three women. There was one case of Type I, one of Type II, three of Type III, and five of Type IV. Three of the ten died. The remaining seven recovered without complications. Case 7 had a prolonged period of resolution. Except for the three fatal cases, they were all of rather mild type. For the purposes of the study this was a disadvantage, as few of the surviving cases showed more than slight cyanosis, and in most a fall of temperature followed shortly after admission to the hospital. It would have been of interest to measure lung volumes in that type of case familiar to clinicians in which there is a relatively small area of consolidation but in which

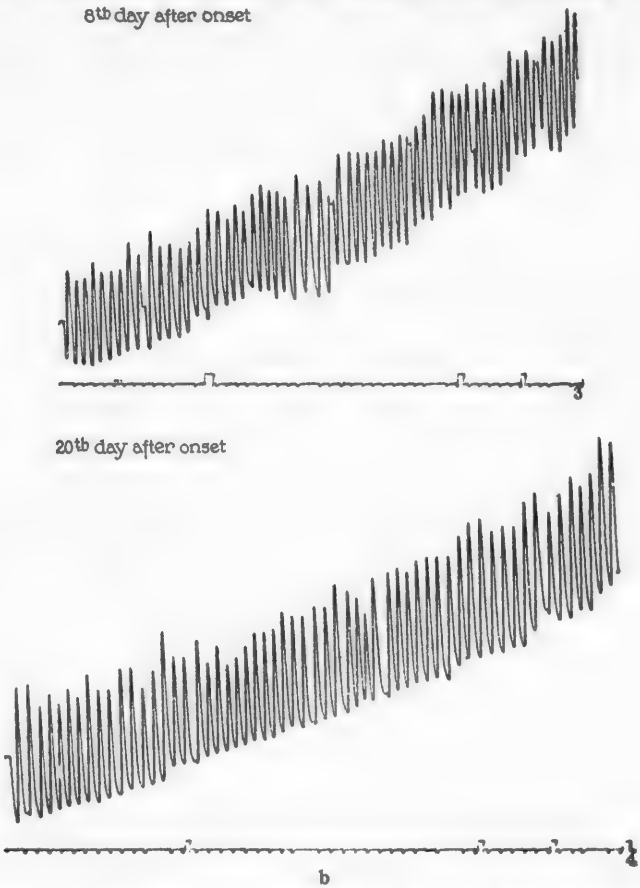
diffuse râles, marked cyanosis, and tachypnea are present; but no such case was available. The lung volumes were never measured on the day of admission, but on the following day when possible, and at succeeding intervals, sometimes daily, sometimes every 2nd or 3rd day, according to the progress of the disease and the change in physical signs. On admission a careful physical and bacteriological examination was made, and on each day of study physical signs and symptoms were



a

TEXT-FIG. 3, *a* and *b*. Tracings made by the mixing spirometer at four different periods of disease in Case 7. The first tracing illustrates pronounced rapid and shallow breathing. Time marker indicates 5 second intervals. Signal indicates time at which samples were taken. The increase in amplitude at the end of tracings 1 and 2 is due to slight CO_2 accumulation resulting from insufficient scrubbing

noted. X-ray pictures were always taken on the day respiratory tests were made. When there was any marked cyanosis, arterial punctures were done and oxygen analyses made with the Van Slyke constant volume apparatus. An effort was made to express cyanosis in a roughly quantitative way by the use of color scales (Flagg (12); Ridgway (13)). Though these may fairly accurately describe the color of the part, they cannot in pneumonia patients be used with any quantitative accuracy because of the difference in skin color and capillary dilatation in various



b

of expired air. The upward slope of each tracing represents the rate of O_2 absorption, which is notably greatest in tracing 1. The factor for the spirometer is 4.82 cm. excursion of the bell for 1 liter. The scale represents centimeters reduced proportionately to the tracing.

patients and in the same patient from day to day. We have had, therefore, to resort to the unsatisfactory method of describing degrees of cyanosis with plus signs.

TABLE I.

Name.	Sex.	Functional residual air.	Mean.	Average deviation.	Per cent deviation.
		liters	*liters*	*liters*	
D.	M.	3.18 3.24 3.17	3.20	±0.03	±1.0
M.	"	3.06 3.18 2.89	3.04	±0.10	±0.33
P.	"	2.76 2.73 2.68	2.72	±0.03	±1.0
Br.	"	2.60 2.61 2.65	2.62	±0.02	±0.66
L.	"	2.53 2.51	2.52	±0.01	±0.50
B.	"	2.25 2.12 2.26 2.38	2.25	±0.09	±2.25
T.	"	2.27 2.28	2.28	±0.01	±0.50
S.	"	1.83 1.95	1.89	±0.06	±3.0
C.	"	1.77 1.69 1.67	1.71	±0.04	±1.33
N.	F.	1.53 1.53	1.53	±0.00	±0.0
Average.....					±1.0

Normal Subjects.

For normal subjects we used physicians and laboratory technicians, all in a good state of health. There were nine men and four women. Functional residual air and pulmonary ventilation were measured with the same technique that was used on the patients. Each subject was required to lie flat on his back in bed for 20 minutes before an observation was made. Physicians are ordinarily very poor subjects for respiratory tests because of consciousness of their breathing during the tests, so that the error in the normal series is probably greater than in the pathological. Furthermore, the normal series was studied first, and with time the accuracy of the technique greatly improved.

Table I shows the average deviation from the mean in repeated determinations of functional residual air in normal subjects. All lung volume determinations in this table were made at rest and with the subjects in the horizontal position.

It follows from the figures given in the table that a variation of ± 1 per cent in the lung volume measurements of patients suffering from pneumonia is to be regarded as without significance, since it is within the normal range. We have arbitrarily taken approximately ± 5 per cent as a variation assuredly significant of a pathological rather than a physiological change. This is borne out by the observations on the patients.

It was obviously desirable to have some method of predicting the volume of the functional residual air normal to the patients, so that at any time one could tell how far the observed lung volume deviated therefrom. In the cases that recovered, it was easy to establish the patient's normal by making successive determinations during convalescence and a month or so after discharge from the hospital until constant findings were obtained. An effort was made to correlate the volume of the functional residual air with some physical measurement, such as height, weight, surface area, or chest volume (Lundsgaard and Van Slyke (14)). This was unsuccessful, as is brought out in Table II, in which the cases are arranged in a series according to the volume of the functional residual air, while for each case the other body measurements are given.

The table shows at a glance that functional residual air cannot with any exactness be derived from height, weight, surface area, or chest volume. Take, for example, the two individuals T. and S., who are

approximately of the same height, weight, and surface area. Notwithstanding this, the volume of the functional residual air in the one case differs from that in the other by about 400 cc. Or take B. and

TABLE II.

Name.	Sex.	Functional residual air.	Height.	Weight.	Surface area.	Chest volume.
		liters	*cm.*	*kg.*	*sq. m.*	*cc.*
D.	M.	3.20	178.6	63.0	1.80	8,660
M.	"	3.04	191.3	87.1	2.16	11,640
P.	"	2.72	180.6	77.4	1.92	10,350
Br.	"	2.62	179.2	66.2	1.84	7,260
L.	"	2.52	182.0	65.5	1.84	8,180
R.	F.	2.32	170.2	70.0	1.81	
T.	M.	2.28	167.0	65.0	1.73	7,780
B.	"	2.25	180.9	79.5	2.00	10,070
S.	"	1.89	169.0	67.0	1.77	6,550
H.	F.	1.72	159.6	52.1	1.52	
C.	M.	1.71	161.2	58.6	1.62	6,740
N.	F.	1.53	153.0	60.1	1.57	
McD.	"	1.37	165.3	58.3	1.65	

TABLE III.

Name.	Sex.	Functional residual air.		
		Lying.	Sitting.	Difference.
		liters	*liters*	*liters*
D.	M.	3.20	4.17	0.97
M.	"	3.04	4.29	1.25
P.	"	2.72	4.00	1.28
Br.	"	2.62	2.99	0.37
L.	"	2.52	3.21	0.69
B.	"	2.25	3.19	0.94
T.	"	2.28	3.09	0.81
S.	"	1.89	2.29	0.40
C.	"	1.71	2.25	0.54

T., the volumes of whose functional residual air are within 30 cc. of each other,—less than the experimental error,—and yet they are of quite different shape and size.

The fact that functional residual air could not be definitely correlated with any of these physical measurements suggested that the

level of the diaphragm was the determining factor, and this idea was strengthened by the observation that some of the broad, deep chested, muscular individuals had relatively smaller lung volumes than long chested subjects with relaxed abdominal walls. If the level of the diaphragm determined the volume of the functional residual air, this should shift with a change in body position, and there should be an increase in the functional residual air with alteration from the horizontal to the vertical position, a fact brought out in Table III.

It will be seen that in every instance the functional residual air was greater in the sitting than in the lying position.

Fig. 1 shows an x-ray photograph of Subject P. taken in the lying and in the sitting position. The pictures were exposed as nearly as possible at the end of a normal expiration.

These pictures show that the level of the diaphragm drops after sitting upright and the heart assumes a narrower shape and a more vertical position, which undoubtedly explains the increase in size of lung volume. It seemed obvious from this that no external measurement can be expected to give information about the level of the diaphragm, and the volume of the functional residual air. An attempt to predict the latter from measurements of the area of the lung shadow on the x-ray films, while fairly satisfactory for normal individuals, did not prove so for the pneumonia patients.

Pneumonia Patients.

The changes occurring in the lung volumes of the pneumonia patients are shown in Text-figs. 4 to 13. These charts show the clinical course of the disease as evidenced by the maximum daily temperature, pulse, and respiratory rate. The curve for the average respiratory rate represents the rate for 24 hours on the day of lung volume determination. It will be noticed that, for the reasons stated above, we have not charted the respiratory rate, depth, and minute volume instrumentally recorded, but have preferred to depend upon the respiratory rate counted in the wards. These charts are arranged from a point of view of duration of active disease (fever, rapid pulse, and respirations) after admission to the hospital. Each one is accompanied by a case protocol. The first six cases (Nos. 9, 6, 11, 2, 7, 3; Text-figs. 4 to 9) all show an almost immediate critical fall in tempera-

ture, pulse, and respiration after admission. In Cases 9, 6, 11, and 2 (Text-figs. 4 to 7), the simultaneous increase in lung volume with this critical fall is well demonstrated. In Cases 7 and 3 (Text-figs. 8 and 9), an initial decrease in lung volume was observed (accompanying a spread in physical signs) which occurred in both instances when the patients were still febrile and exhibited accelerated heart rate and rapid breathing, the increase in lung volume not occurring until the more or less critical drop of temperature, pulse, and respirations. The concomitant disappearance of cyanosis should be noted, as well.

In Case 8 (Text-fig. 10), a persistence of fever will be observed from the 3rd to the 8th day after onset. During this period three lung volume determinations were made on the 4th, 6th, and 8th days after onset, respectively. A progressive reduction is seen, accompanied by persistence of cyanosis. This patient had a prolonged period of convalescence with persistent cough and sputum and presence of râles in the right chest. The marked fluctuation in lung volume is shown in Text-fig. 10. In connection with this it should be stated that this case and several others, in which there was marked fluctuation in lung volume, were accompanied in resolution by profuse expectoration, and those in which the lung volume remained constant had relatively scant sputum production.

Cases 4 and 10 (Text-figs. 11 and 12) terminated fatally. In these there was no fall of temperature, pulse, or respiration, and *no increase but a decrease* in the volume of the functional residual air was observed. Likewise, cyanosis persisted. Case 5 (Text-fig. 13) is presented, in spite of the fact that only one lung volume determination was made, because of the markedly rapid respiratory rate (60 to the minute) and the pronounced oxygen unsaturation of the arterial blood (32 volumes per cent). This case had the smallest volume of functional residual air of any of the series, only 990 cc. in an individual in whom one might anticipate at least 2 to 2.5 liters. The rapid breathing and extreme cyanosis should be noted in association with this marked reduction in lung volume. From an examination of Text-figs. 4 to 13 the conclusion seems justified that functional residual air varies synchronously with the course of the disease as evidenced by temperature, pulse, and respiratory rate. When these fall, the volume of the functional residual air increases. When fever, rapid heart rate,

and rapid respirations persist, the functional residual air either progressively diminishes or remains fixed. Cyanosis similarly in this series of cases disappeared when lung volume began to increase, and was most intense in the three fatal cases in which there were very rapid and shallow breathing and a marked reduction in functional residual air.

It may be questioned at this point whether decrease in functional residual air represents a spread in the pulmonary process and increase is evidence of resolution. We are not prepared to answer this question. The functional residual air represents the volume of air enclosed in the lungs at the end of spontaneous expiration. When this volume is diminished it may be so from a variety of causes other than obliteration of air sacs by consolidation, such as vascular congestion or nervous changes due to modifications in the Hering-Breuer reflex. A close study of the relation of physical signs, x-ray pictures, and functional residual air at different stages of the disease shows them in most instances to vary synchronously and strongly suggests that they all represent different manifestations of the same phenomenon; namely, consolidation and resolution. Fig. 2 illustrates the changes occurring in physical signs, x-ray shadows, and functional residual air observed in Case 8.

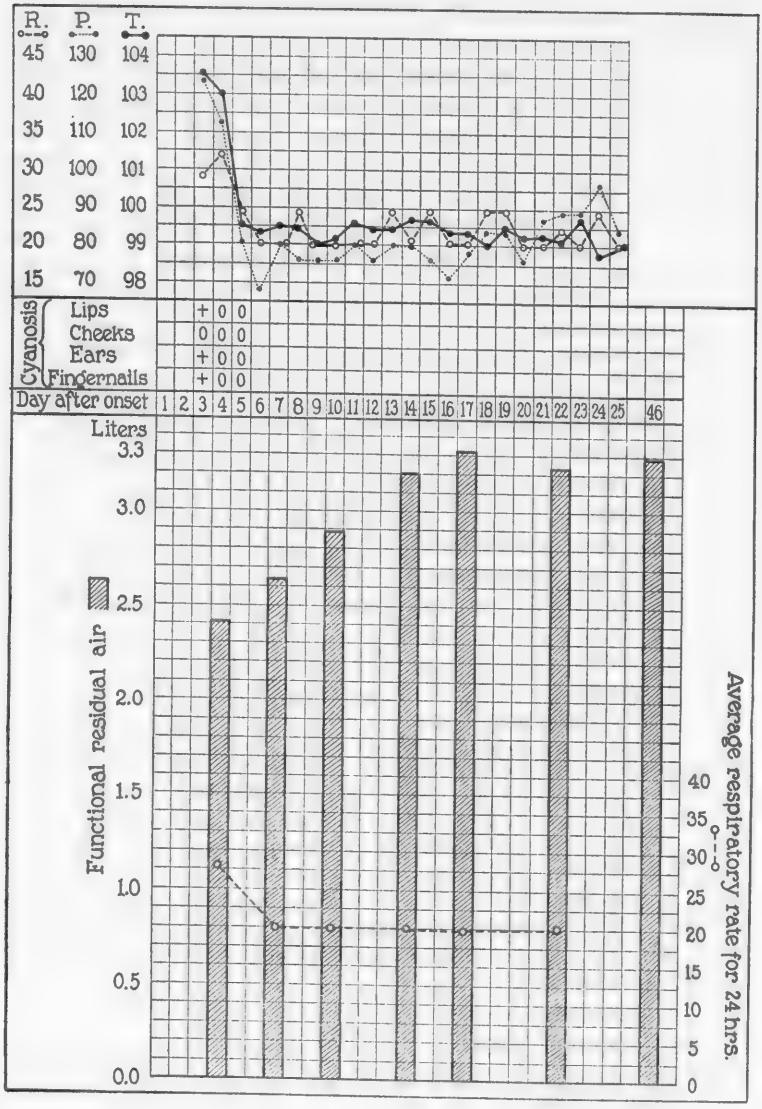
Case 9 (*Text-Fig. 4*).—J. M. Diagnosis: Lobar pneumonia, Type IV. Admitted April 26, 1923, 3 days after onset. Symptoms on admission: Pain in left chest. Headache and fever. Cough with bloody sputum. Physical signs on admission: Dullness over left lower lobe. Coarse moist râles below inferior angle of left scapula. Slight cyanosis of lips, finger-nails, and ears. Some abdominal tenderness. Rusty, blood-streaked sputum.

Clinical Course.—4th day after onset: Moderate cyanosis of lips and finger-nails. Considerable cough with frothy, rusty sputum. Abdominal tenderness in epigastrium. No distention. Dullness over left lower lobe with coarse moist râles.

5th day after onset: No cyanosis. Coughing less severe; copious, frothy, purulent sputum. No abdominal pain. Dullness and coarse moist râles over left lower lobe.

6th day after onset: Occasional cough with scant, frothy sputum. Resonance clearing over left lower lobe; coarse moist râles.

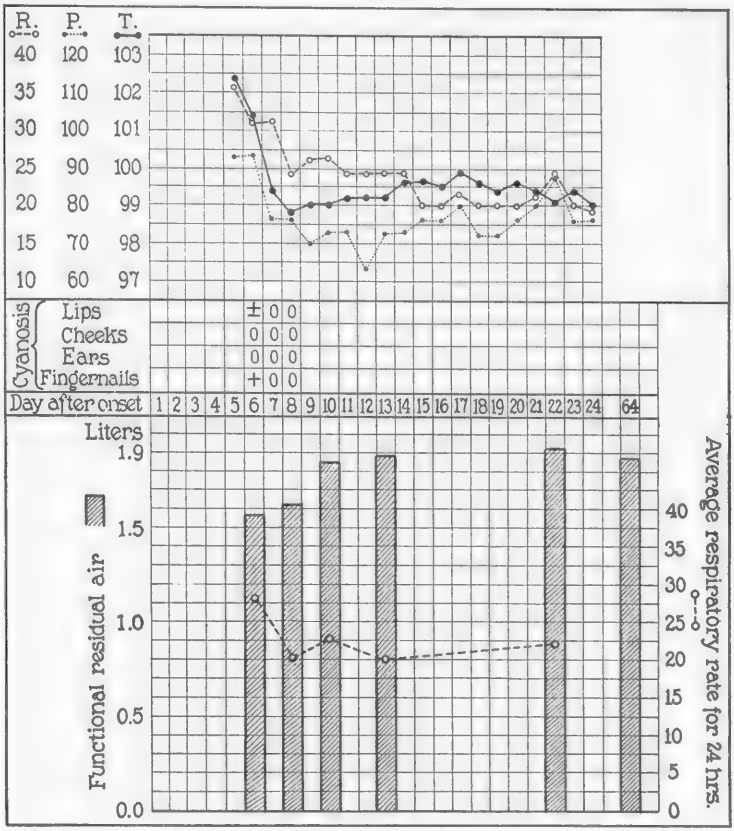
7th day after onset: Slight pain in lower left chest on coughing. Occasional cough with scant, purulent sputum. Impaired resonance with coarse moist râles over left lower lobe; breath sounds clearing.



TEXT-FIG. 4. Case 9.

10th day after onset: Very occasional cough with scant sputum. Resonance only very slightly impaired with distant breath sounds. No râles over left lower lobe.

14th day after onset: No cough or expectoration. Resonance still slightly impaired over left lower lobe; a few fine crackles at the left base.



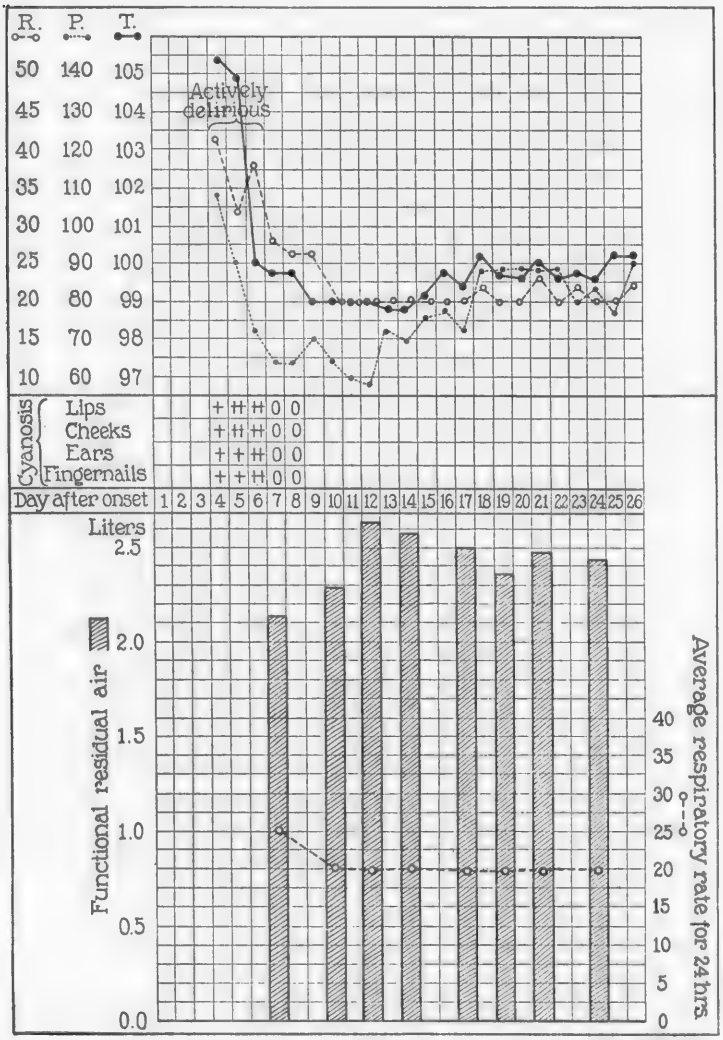
TEXT-FIG. 5. Case 6.

17th day after onset: No cough. Slightly impaired resonance with distant breath sounds over the left base. No râles.

22nd day after onset: No cough. Resonance clear over left base. Distant breath sounds but no râles heard at left base.

25th day after onset: Discharged. No cough. Still distant breath sounds at left base; normal breathing; no râles. Resolution complete.

46th day after onset: Normal.



TEXT-FIG. 6. Case 11.

Case 6 (*Text-Fig. 5*).— J. D. Diagnosis: Lobular pneumonia, Type IV. Admitted April 15, 1923, 5 days after onset. Symptoms on admission: Pain in right chest. Cough and expectoration. Physical signs on admission: Patch of impaired resonance at right base. Moist râles. Dry friction rub below right scapula. Very slight cyanosis of lips and finger-nails. Cough, with frothy, yellowish sputum.

Clinical Course.—6th day after onset: Pain in right posterior chest on breathing. Slight cyanosis of lips and finger-nails. Slight cough with scant sputum. Impaired resonance with moist râles in lower right posterior chest. White blood cells 23,000.

8th day after onset: Slight cough with scant, purulent sputum. Slightly impaired reasonance at right base; showers of moist râles. White blood cells 9,375.

10th day after onset: No cough. Very slightly impaired resonance in right paravertebral line with a few moist râles.

13th day after onset: No cough. Very slightly impaired resonance in right paravertebral line with a few fine râles.

22nd day after onset: No cough. Resonance clear at right base. Occasional fine râle in right paravertebral line after forced cough.

64th day after onset: Normal.

Case 11 (*Text-Fig. 6*).—R. L. Diagnosis: Lobar pneumonia, Type IV. Admitted May 29, 1923, 4 days after onset. Symptoms on admission: Cough. Chilly sensations. Headache. Physical signs on admission: Impaired resonance in upper right chest. Sticky râles to level of fourth rib in right chest. Moderate cyanosis of cheeks, lips, and finger-nails. Cough, with scant, frothy, rusty sputum. Mental confusion.

Clinical Course.—4th and 5th days after onset: Patient quite irrational and actively delirious, requiring restraint. Cough troublesome with scant, frothy, rusty sputum. Moderate cyanosis of cheeks, lips, and finger-tips.

6th day after onset: Loquacious and at times irrational. Considerable unproductive cough. Marked cyanosis of cheeks, lips, and hands after coughing. Consolidation of right upper lobe with moist râles.

7th day after onset: Rational; slight pain in lower right chest. Considerable unproductive cough. Consolidation of right upper lobe with dullness, moist râles, and bronchial breathing.

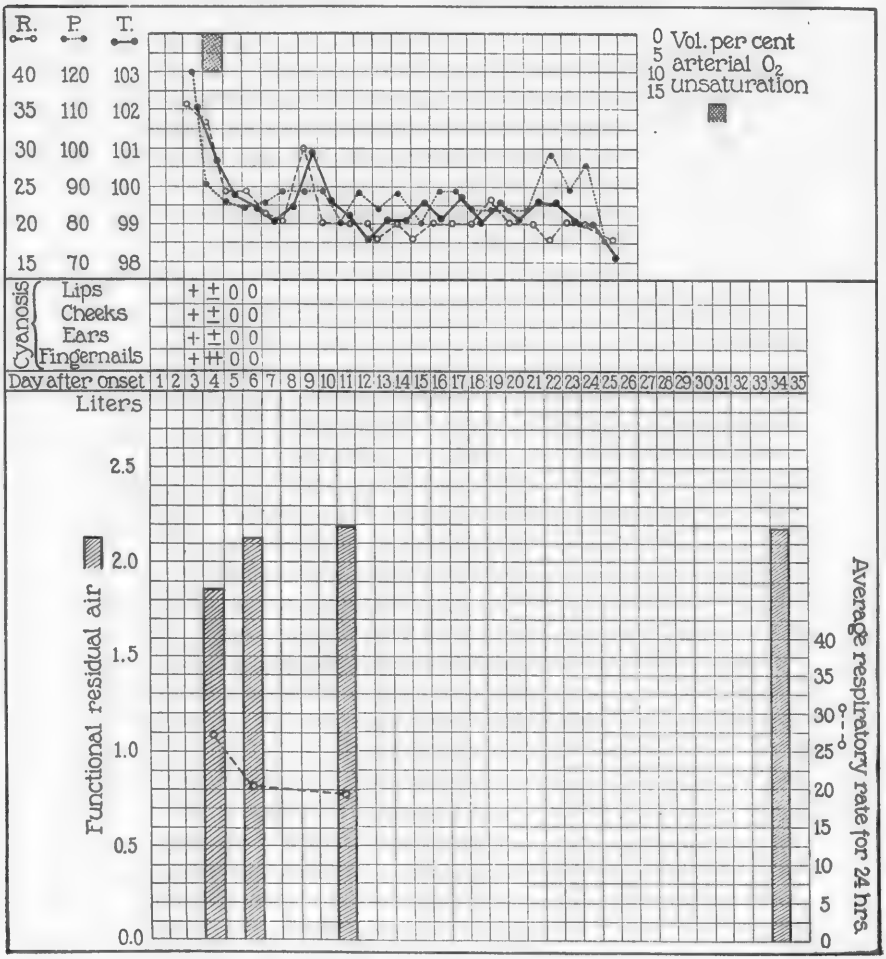
10th day after onset: Occasional unproductive cough. Showers of coarse, bubbling râles over upper right lobe with increased voice sounds. Resonance only slightly impaired.

12th day after onset: No cough. Resonance clearing over right upper lobe. Scattered fine râles over right apex.

14th day after onset: No cough. Resonance only slightly impaired over upper right lobe. Few fine râles at right apex; voice sounds normal.

17th, 19th, 21st, and 24th days after onset: No cough. Signs over right upper lobe show resolution to be complete. No râles.

Case 2 (*Text-Fig. 7*).—O. J. Diagnosis: Lobar pneumonia, Type IV; acute fibrinous pleurisy (right). Admitted February 6, 1923, 3rd day after onset. Symp-

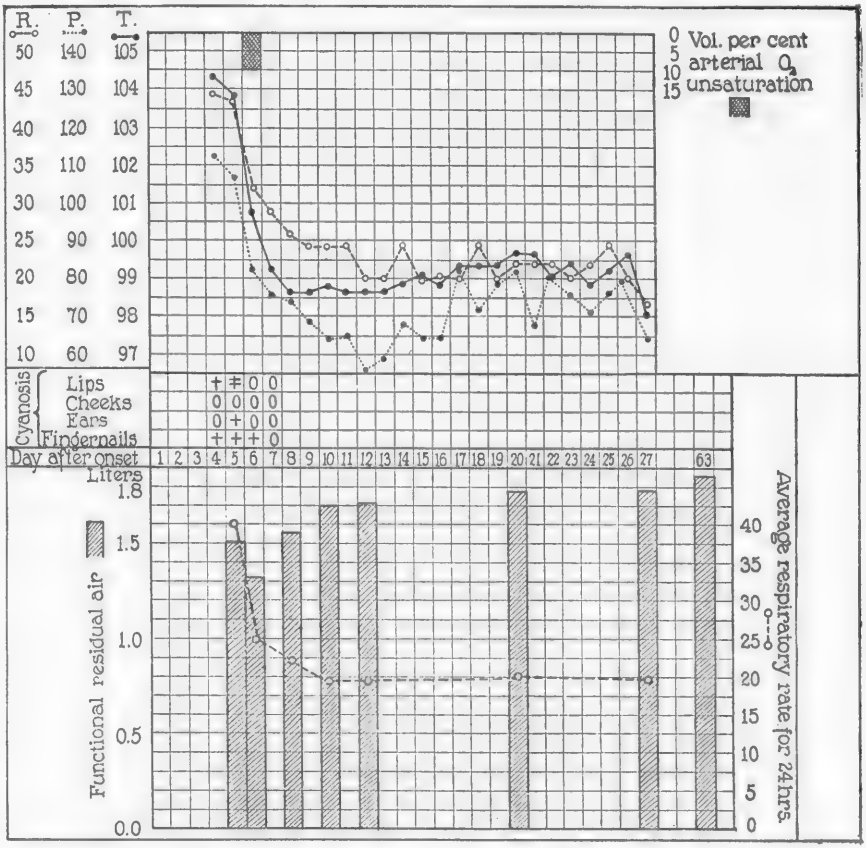


TEXT-FIG. 7. Case 2.

toms on admission: Pain in the right chest. Cough. Blood-streaked sputum. Physical signs on admission: Posteriorly the excursion of the lower half of the right chest is restricted. Resonance impaired below the seventh right rib pos-

teriorly, with bronchial breathing and moist râles. Dry friction rub anteriorly at the level of the right nipple. Slight cyanosis of the lips, finger-tips, ear lobes, and cheeks. Moderate cough with tenacious, rusty expectoration.

Clinical Course.—4th day after onset: Slight pain in the anterior right chest on coughing. Cough less severe, expectoration rusty and less copious. Impaired



TEXT-FIG. 8. Case 7.

resonance below the sixth rib of the right posterior chest with coarse râles and bronchial breathing. Perspiration profuse. Slight cyanosis of the lips, finger-nails, ears, and cheeks. White blood cells 28,125. Arterial oxygen: content 18.9 volumes per cent; capacity 20.7 volumes per cent; arterial unsaturation 10 per cent.

6th day after onset: Moderate cough with scant, brown, purulent sputum. Moderate perspiration. No cyanosis. Slightly impaired reso. nce with scattered râles in the right paravertebral line about the midscapular n. A dry friction rub is still heard about the right nipple area anteriorly.

11th day after onset: No cough or expectoration. A few scattered moist râles are heard along the right paravertebral line.

34th day after onset: Patient has been discharged for 9 days and returns for lung volume determination. He has no complaints and is gaining weight and strength. Physical examination shows apparently a normal right lung.

Case 7 (*Text-Fig. 8*).—M. W. Diagnosis: Lobar pneumonia, Type II; acute pleurisy. Admitted April 17, 1923, 4 days after onset. Symptoms on admission: Pain in left chest. Shortness of breath. Cough. Physical signs on admission: Dullness and râles below inferior angle of left scapula. Dry pleural rub in left axilla. Slight cyanosis of lips, cheeks, and finger-nails. Cough, with rusty, bloody sputum.

Clinical Course.—5th day after onset: Pain in left chest on coughing or breathing. Definite cyanosis of lips, cheeks, and finger-nails. Slight cough with scant, rusty sputum. Slight abdominal distention. Consolidation of left lower lobe below the inferior angle of the left scapula. Moist râles heard above lower left lobe, and a few dry râles at the right base. White blood cells 10,625.

6th day after onset: Slight cyanosis of lips and finger-nails. Coughing very little, unproductive. No abdominal distention. Signs of consolidation with râles below inferior angle of left scapula. Oxygen content of arterial blood 17.5 volumes per cent; capacity 19.5 volumes per cent; unsaturation 10 per cent.

8th day after onset: No cyanosis. Moderate cough with scant, purulent sputum. Impaired resonance over left lower lobe; coarse, bubbling, moist râles over left lower lobe.

10th day after onset: Very slight unproductive cough. Slightly impaired resonance over left lower lobe, breath sounds more normal, with moist râles.

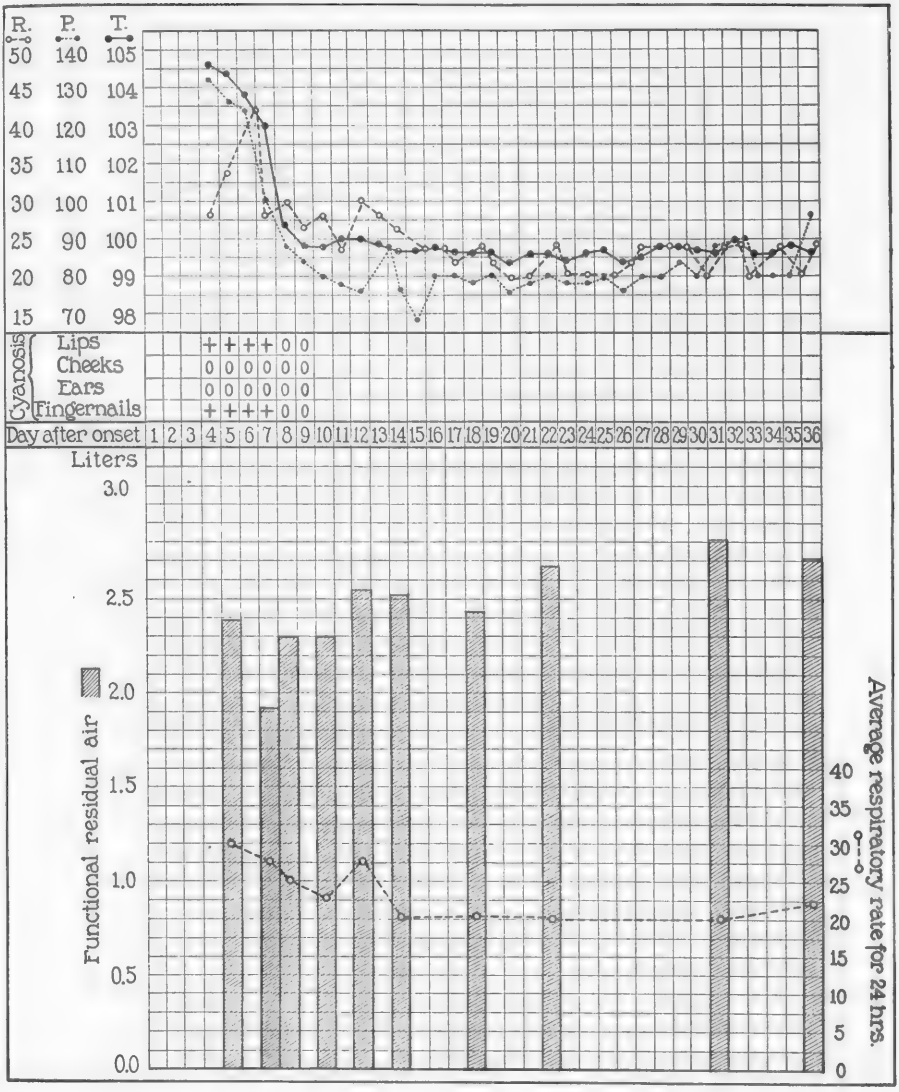
12th day after onset: Occasional unproductive cough. Resonance only slightly impaired over the left lower lobe; scattered fine crackles at left base.

20th day after onset: Resonance clear at left base. No râles. No cough.

27th and 63rd days after onset: Normal.

Case 3 (*Text-Fig. 9*).—W. G. Diagnosis: Lobar pneumonia, Type I. Admitted March 23, 1923, 4 days after onset. Symptoms on admission: Headache. Pain in left chest. Cough. Physical signs on admission: Dullness in lower left chest. Râles below the fourth rib left posterior chest. Slight cyanosis of lips and finger-nails. Cough, with rusty, blood-tinged sputum.

Clinical Course.—4th day after onset: Following intracutaneous horse serum test patient developed marked anaphylactic shock symptoms with dyspnea, cyanosis, accelerated pulse, cough, widespread urticaria, nausea, vomiting, and diarrhea.



TEXT-FIG. 9. Case 3.

TEXT-FIG. 10. Case 8.

5th day after onset: Irrational and restless. Slight cyanosis of lips and finger-nails. Cough, with large amount of frothy sputum. Consolidation of entire left lower lobe. White blood cells 25,625. Blood culture positive, Type I pneumococcus.

7th day after onset: Slight cyanosis of lips and finger nails. Frequent cough, with scant, purulent, blood-streaked sputum. Consolidation of left lower lobe with a few moist râles. White blood cells 12,812.

8th day after onset: Frequent cough with small amount of frothy, rusty sputum. Resonance clearing over left lower lobe; very few moist râles heard.

10th day after onset: Slight cough with very scant, purulent sputum. Consolidation still present in left lower lobe with very few moist râles. Slight pain in left chest.

12th day after onset: Respiratory rate increased. Occasional cough with scant expectoration.

14th day after onset: Occasional unproductive cough. Resonance impaired over left lower lobe. Scattered coarse, moist râles over left lower lobe.

18th day after onset: Slight cough, no expectoration. Impaired resonance over left lower lobe with scattered fine râles.

22nd day after onset: No cough or expectoration. Very slight impaired resonance over left lower lobe, few fine râles, normal breath sounds.

31st day after onset: No cough. Resonance clear, no râles. Resolution complete.

36th day after onset: Normal.

Case 8 (*Text-Fig. 10*).—C.M. Diagnosis: Lobar pneumonia, Type III. Admitted April 25, 1923, 3 days after onset. Symptoms on admission. Pain in right shoulder. Cough with blood-streaked sputum. Headache. Physical signs on admission: Dullness and moist râles and increased voice sounds over upper right lobe. Few moist râles at left base. Considerable cough with small amount of tenacious, rusty, bloody sputum. Slight cyanosis of lips, ears, and finger-nails.

Clinical Course.—4th day after onset: Severe headache. Moderate cyanosis of finger-nails and lips. Considerable cough with tenacious, rusty sputum. Moderate abdominal tympanites. Dullness and moist râles in upper right lobe. White blood cells 21,250.

6th day after onset: Shortness of breath. Pain in right chest on coughing. Moderate cyanosis of lips and finger-nails. Cough less severe; sputum bloody and rusty. Moderate abdominal distention. Dullness and moist râles, apparently spread to upper part of middle and lower right lobes. White blood cells 21,875.

8th day after onset: Crisis during the early morning followed by profuse perspiration. Considerable coughing with tenacious, rusty, blood-streaked sputum. Resonance clearing in right upper lobe with coarse bubbling râles and dullness of middle and right lower lobes with bronchial breathing.

10th day after onset: Moderate cough with yellowish, purulent sputum. Resonance clearing from above downward to the right base with widespread, coarse bubbling râles throughout.

11th day after onset: Feeling of tightness in right side of chest on taking a deep breath. Moderate cough with tenacious, frothy, purulent sputum. Resonance clearing to third rib anteriorly and seventh rib posteriorly, with coarse bubbling râles, while below are dullness and bronchial breathing.

12th day after onset: Moderate cough with yellowish, purulent sputum. Resonance clearing over right chest; bronchial breathing disappeared. Coarse bubbling râles throughout.

13th day after onset: Moderate cough with scant, tenacious, purulent sputum. Resonance slightly impaired over posterior right chest; widespread moist râles with a few dry râles heard throughout right chest.

14th day after onset: Pain on pressure over the third and fourth ribs anteriorly and on taking a deep breath. Coughing moderately with tenacious, slightly rusty, and blood-streaked sputum today. Physical signs same as on last day.

16th day after onset: Occasional cough with tenacious, purulent, slightly blood-streaked sputum. Slightly impaired resonance over the right chest; breath sounds faint; showers of fine moist râles over right chest.

17th day after onset: Occasional cough with few tenacious globules of purulent sputum. Signs unchanged.

23rd day after onset: Occasional cough with a few globules of purulent, yellowish sputum. Slightly impaired resonance with showers of fine râles in the right axilla.

27th day after onset: Occasional cough with scant purulent sputum. Showers of fine râles with distant breath sounds in right axilla.

30th day after onset: Still occasional cough with small amount of sputum. Slightly impaired resonance with showers of fine râles in right axilla and radiating posteriorly and anteriorly.

34th day after onset: Very occasional unproductive cough. Numerous fine moist râles with slightly impaired resonance in right axilla.

40th day after onset: Slight unproductive cough. Fine moist râles persist in the right axilla and over the lateral border of middle right lobe.

45th day after onset: Occasional unproductive cough. A very occasional fine moist râle is heard in the right axilla after a deep breath.

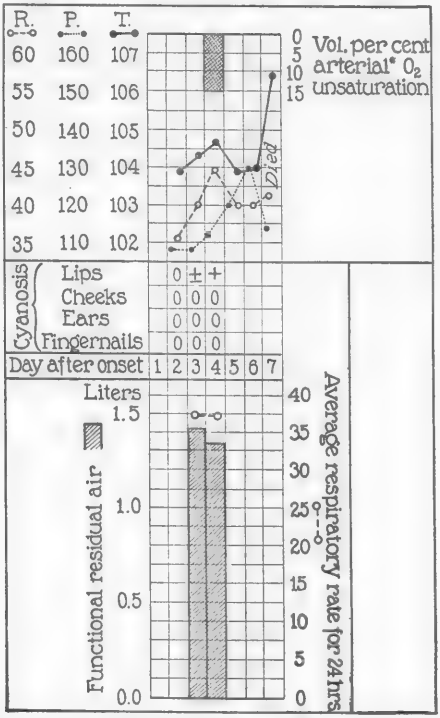
Case 4 (*Text-Fig. 11*).—K. O. Diagnosis: Lobar pneumonia, Type III. Admitted March 13, 1923, 2 days after onset. Symptoms on admission: Cough. Fever. Coryza. Physical signs on admission: Impaired resonance below the seventh rib, right posterior, with suppressed breath sounds and moist râles. Respirations rapid and shallow. Slight unproductive cough.

Clinical Course.—3rd day after onset: Troublesome cough with scant amount of tenacious, rusty, bloody sputum. No cyanosis. Dullness, bronchial breathing, and increased voice sounds over right lower lobe. A few râles heard at left base posteriorly. White blood cells 21,875.

4th day after onset: No cyanosis. Moderate cough with scant, rusty sputum. Dullness and definite consolidation of whole right lower lobe. A few râles heard at the left base. White blood cells 23,125. Blood from hand vein: oxygen content

13.95 volumes per cent; oxygen capacity 16.35 volumes per cent; oxygen unsaturation 15 per cent.

7th day after onset: Mentally confused and toxic. Weak. Mucus rattle in throat. Slight unproductive cough. Increasing cyanosis of lips and fingers. Considerable abdominal distention. Complete dullness over entire right chest; coarse palpable rhonchi over both sides. White blood cells 26,230. Blood culture negative. Death.



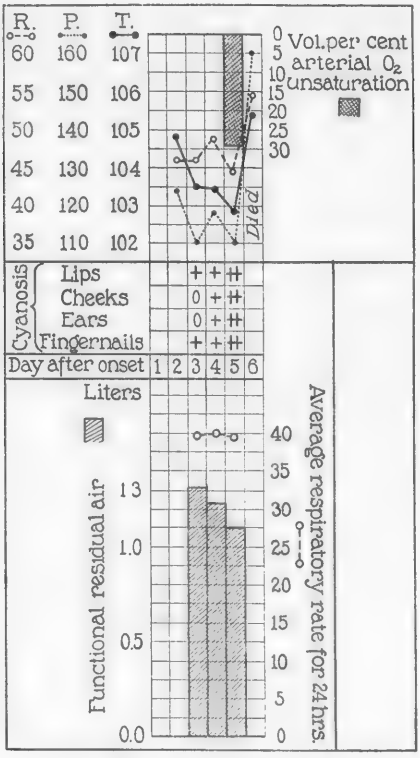
*Blood was drawn from the hand vein. This has been shown recently (Goldschmidt, S., personal communication) to agree closely in its oxygen content with arterial blood.

TEXT-FIG. 11. Case 4.

Case 10 (*Text-Fig. 12*).—J. McG. Diagnosis: Lobar pneumonia, Type III, with Pneumococcus Type III bacteremia. Admitted May 15, 1923, 3 days after onset. Symptoms on admission: Cough with blood-streaked expectoration. Pain in left chest. Fever. Physical signs on admission: Restricted movement of

left lower chest with dullness, moist râles, and bronchial breathing over the left lower lobe. Slight cyanosis of lips, cheeks, and finger-nails. Cough with rusty, bloody sputum.

Clinical Course.—3rd day after onset: Headache and cough. Moderate cyanosis of cheeks, lips, and finger-nails. Slight cough with tenacious, rusty, bloody sputum. Mucus rattle in throat. Consolidation of left lower lobe; diffuse

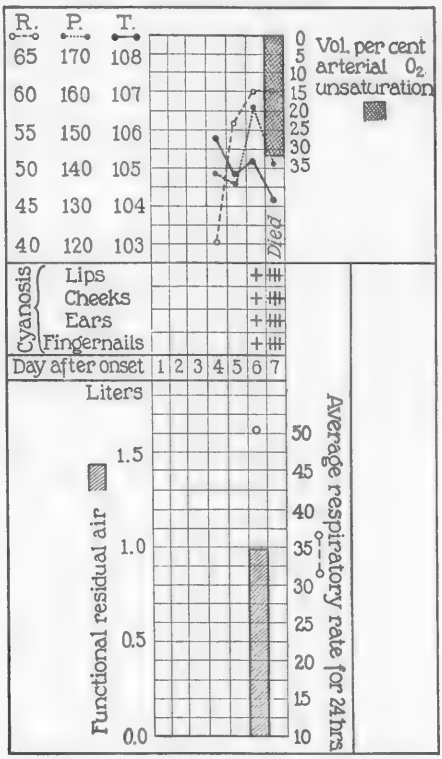


TEXT-FIG. 12. Case 10.

palpable rhonchi over both lungs. Few râles at right base posteriorly and upper left chest. White blood cells 31,875.

4th day after onset: Respirations rapid and shallow, mostly abdominal in character. Moderate cyanosis of cheeks, lips, ears, and hands. Moderate cough with frothy, brown sputum. Consolidation of whole left chest with palpable rhonchi over both lungs. Few râles at right base. White blood cells 21,250.

5th day after onset: Pain in nape of neck and lower lumbar region. Respirations rapid and shallow, mostly abdominal. Marked cyanosis of cheeks, lips, ears, and hands. Moderate cough with frothy, brownish sputum. Consolidation of left lung complete, with dullness and râles. A patch of dullness and râles at right base posteriorly. White blood cells 26,250. Blood from hand vein: oxygen content 13 volumes per cent; capacity 18.1 volumes per cent; unsaturation 28 per cent. Death.



TEXT-FIG. 13. Case 5.

Case 5 (*Text-Fig. 13*).—E. M. Diagnosis: Lobar pneumonia, Type IV. Admitted March 24, 1923, 4 days after onset. Symptoms on admission: Pain in left chest. Cough with blood-tinged sputum. Fever. Physical signs on admission: Dullness over left lower lobe. Moist râles over left lower lobe. Breathing rapid, thoracoabdominal in type. Moderate abdominal distention. Cough with scant, rusty, blood-streaked sputum.

Clinical Course.—6th day after onset: Pain in left chest on coughing and breathing. Cyanosis of lips, cheeks, ears, and finger-nails. Frequent unproductive cough. Moderate abdominal distention. Consolidation with dullness and râles over whole left chest. Right chest shows areas of dullness and diffuse moist râles. White blood cells 5,625. Arterial blood: oxygen content 10.6 volumes per cent; oxygen capacity 15.6 volumes per cent; oxygen unsaturation 32 per cent. Death.

Addendum.—Since the preparation of this article Levy (15) has published a paper showing that the size of the heart in pneumonia increases during the course of the disease and returns to a constant gradually. Comparison of his figures[1] with ours is of interest in respect to the cardiac changes which apparently must occur simultaneously with those in lung volume. If one were to superimpose our diagrams on his one would be led to believe that cardiac dilatation occurred at the same time that lung volume was reduced.

SUMMARY AND CONCLUSIONS.

1. The functional residual air (defined as the lung volume at the end of normal expiration) has been determined in a series of normal individuals and in ten patients with lobar pneumonia at different stages of the disease.

2. The rate, depth, and minute volume of respirations were measured in the same individuals by a graphic method.

3. When appreciable cyanosis was present the oxygen content and capacity of the arterial blood were determined.

4. A constant relationship has been found to exist between the persistence and disappearance of symptoms (fever, accelerated heart rate, rapid and shallow breathing, cyanosis) and fluctuations of the functional residual air. When these symptoms persisted the functional residual air decreased; during their disappearance the volume of the functional residual air rose towards normal. The rise was detected soon after the crisis.

5. A close parallelism has been observed also between alterations in radiographic shadow, physical signs, and the volume of the functional residual air. The lung volume, measured at normal expiration, is diminished during the persistence of pathological signs in the lungs, and returns to normal as the pathological signs disappear. The average time required, in cases which recovered, for the functional residual air to become constant was 11 to 12 days, counting from the onset of the disease.

[1] Levy (15), Figs. 5, 7, and 9.

BIBLIOGRAPHY.

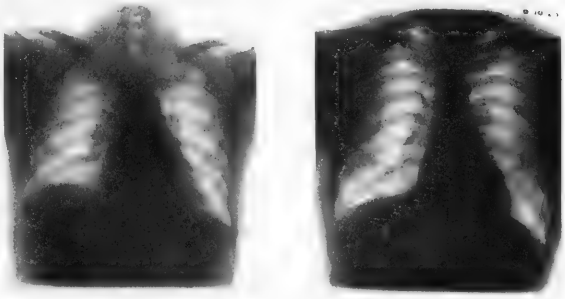
1. Panum, P. L., *Arch. ges. Physiol.*, 1868, i, 125.
2. Bohr, C., *Deutsch. Arch. klin. Med.*, 1906–07, lxxxviii, 385.
3. Lundsgaard, C., and Schierbeck, K., *Acta med. Scand.*, 1923, lviii, 541.
4. Siebeck, R., *Deutsch. Arch. klin. Med.*, 1910, c, 204; 1912, cvii, 252.
5. Krogh, A., in Abderhalden, E., Handbuch der biochemischen Arbeitsmethoden, Berlin and Vienna, 1915, viii, 529.
6. Krogh, A., *Wien. klin. Woch.*, 1922, xxxv, 290.
7. Benedict, F. G., and Collins, W. E., *Boston Med. and Surg. J.*, 1920, clxxxiii, 449.
8. Hendry, M. F., Carpenter, T. M., and Emmes, L. E., *Boston Med. and Surg. J.*, 1919, clxxxi, 285.
9. Roth, P., *Boston Med. and Surg. J.*, 1922, clxxxvi, 491.
10. Van Slyke, D. D., and Binger, C. A. L., *J. Exp. Med.*, 1923, xxxvii, 457.
11. Binger, C. A. L., *J. Exp. Med.*, 1923, xxxviii, 445.
12. Flagg, P. J., *Proc. Soc. Exp. Biol. and Med.*, 1922–23, xx, 1.
13. Ridgway, R., Color standards and color nomenclature, Washington, 1912.
14. Lundsgaard, C., and Van Slyke, D. D., *J. Exp. Med.*, 1918, xxvii, 65.
15. Levy, R. L., *Arch. Int. Med.*, 1923, xxxii, 359.

EXPLANATION OF PLATES 35 AND 36.

FIG. 1. Radiographic pictures of a normal subject (P.) showing changes in position of diaphragm and heart accompanying alteration in the subject's position from lying to sitting. The functional residual air appropriate for the position is indicated.

FIG. 2, *a* to *e*. Diagrams indicating coincident changes in radiographic shadow, physical signs, and functional residual air in a patient at different intervals during the process of consolidation and resolution. The lines indicate impaired resonance; dots indicate the presence of râles.

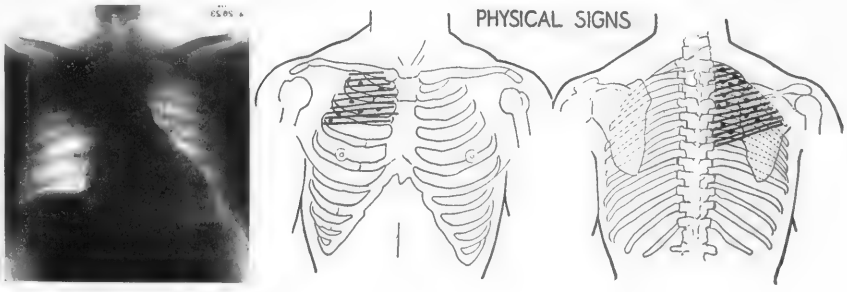
Normal Subject P.



Lying 2.71 liters. Sitting 4.05 liters.

FIG. 1.

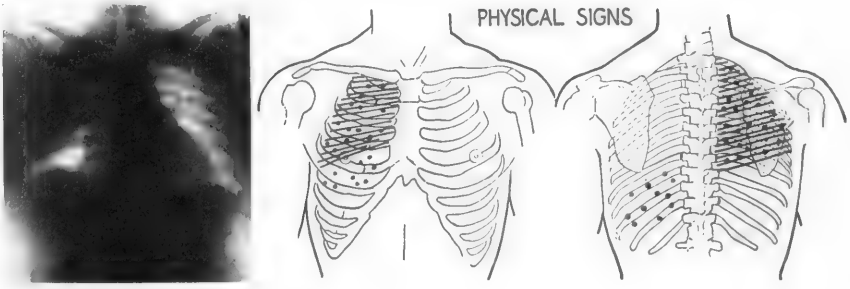
4th day after onset.



1.68 liters functional residual air.

FIG. 2, *a*.

6th day after onset.



1.54 liters functional residual air.

FIG. 2, *b*.

(Binger and Brow: Respiratory mechanism in pneumonia.)

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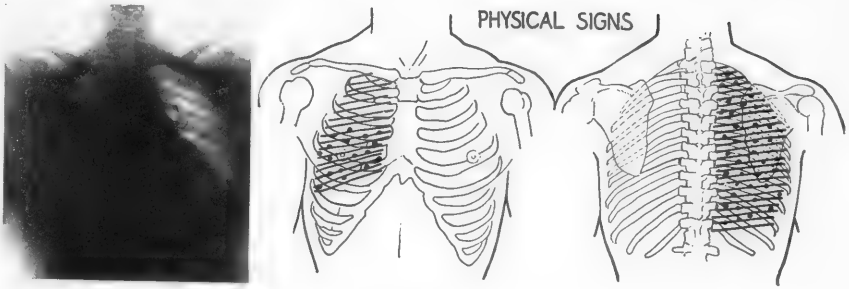
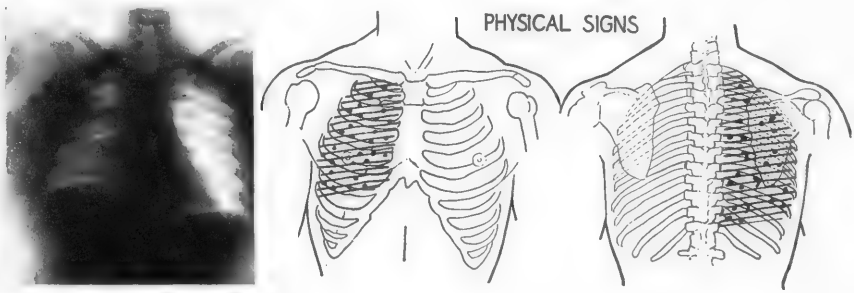
8th day after onset.



1.41 liters functional residual air.

FIG. 2, *c*.

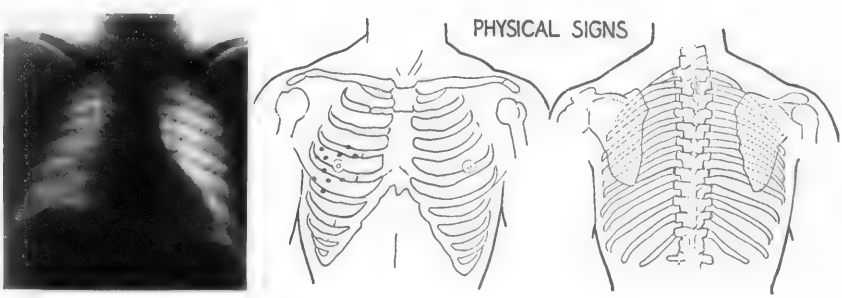
14th day after onset.



1.57 liters functional residual air.

FIG. 2, *d*.

40th day after onset.



1.66 liters functional residual air.

FIG. 2, *e*.

(Binger and Brow: Respiratory mechanism in pneumonia.)

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THE RELATION OF BLOOD PRESSURE TO THE AMOUNT OF RENAL TISSUE.*

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(Received for publication, November 21, 1923.)

The object of this study is to determine the effect on the blood pressure of a marked reduction of renal tissue. In chronic glomerulonephritis, the gradual rise of blood pressure which is usually observed as the disease progresses, has been attributed by some observers to progressive atrophy of renal tissue (Jawein (1)).

The problem has been attacked experimentally by the removal or destruction of a large part of the renal tissue. Pilcher (2) produced renal insufficiency by ligating branches of the renal arteries but obtained no cardiac hypertrophy even after a year. Pässler and Heinike (3) report a marked left ventricular hypertrophy in seven dogs after surgical reduction of kidney parenchyma. A rise in blood pressure in all seven is also recorded. The average increase was 21.5 mm. of mercury. The pressure was measured by means of a cannula in the femoral artery. These experiments have been widely quoted. Janeway (4) ligated branches of the renal arteries in nine dogs. Four of the dogs died. Three of those which died, and two of those which survived showed a slight rise of blood pressure. The pressure was taken by means of a cuff on the foreleg.

Method.

The animal used in these experiments was the rabbit.

Blood Pressure Apparatus.—This instrument has been devised for making repeated determinations of the blood pressure in the central artery of the rabbit's ear. This instrument, which has been previously described (5) does not injure or frighten the rabbit and any

*Read before the Minnesota State Medical Society, October 12, 1923.

desired number of blood pressure readings may be made. Each recorded determination represents an average of from five to ten readings.

When conditions which cause variations of blood pressure, such as fright, temperature, movement, are properly controlled, the readings seldom vary over 20 mm. of mercury when taken at intervals through many weeks. The pressure at any given time, within the artery used, can be determined very accurately, probably within 2 mm. of mercury. When ten consecutive readings are taken there is seldom a difference of over 8 mm. between the highest and the lowest reading.

Operative Procedure.—The operative reduction of the kidney tissue followed closely the technique described by Bradford (6). At the first operation a wedge-shaped piece was removed from the left kidney. At a later date the right kidney was removed. The average weight of the right kidney was 8 gm., and of the piece removed from the left kidney, 2.4 gm. Taking the total weight of both kidneys in each case as twice that of the right, about 65 per cent by weight (61.4 per cent to 66 per cent in individual cases) of the renal tissue was removed. In addition there was some subsequent loss of tissue at the site of the operation in the left kidney. Consequently, the percentage of tissue remaining was probably less than 30 per cent in most instances.

In four rabbits, instead of removing a wedge, several infarcts were produced in the left kidney. This was done by cutting through different areas with loops of silk thread. About 40 to 50 per cent of the kidney was considered infarcted.

Blood Chemistry.—From time to time 5 cc. of blood were withdrawn from an ear vein and analyzed for creatinine and urea nitrogen by the method of Folin and Wu (7).

PROTOCOLS.

1. Feb. 18, 1922. 1.9 gm. of tissue removed from left kidney. Mar. 4, right kidney removed; weight 8.3 gm. Specific gravity of urine, 14 specimens, Apr. 22 to 27, 1011 to 1017; 9 specimens, June 13 to 19, 1014 to 1016. May 18, fundi normal. Death July 1. The results of the chemical and pressure determinations are shown in Table I. A renal insufficiency was present from Mar. 4 to July 1, but the blood pressure remained within normal limits. At post mortem the kidney remnant showed marked atrophy microscopically, due apparently to pressure of scar tissue on the medullary part of the tubules.

2. Apr. 29, 1922. 1.8 gm. of the left kidney removed. June 3, right kidney removed; weight 7.9 gm. A slight albuminuria was present after the first operation. The specific gravity of the urine showed a range of variation of 15 points before the removal of the right kidney, and in 30 specimens taken after the operation one of only 3 to 7 points. There was a little retention of urea nitrogen and creatinine, but only a few observations were made (Table II). The blood pressure was not affected. The fundi were negative on May 18 and Aug. 9.

TABLE I.

Day of experiment.	Creatinine per 100 cc.	Urea N per 100 cc.	Blood pressure.
	mg.	*mg.*	
1			1st operation.
12	1.34		
14			2nd operation.
28	1.6	44.8	
36			70
44			74
46	2.0		
58	3.3	65.0	
63			86
68			85
72	3.7	33.6	
79	3.35		
81			77
88			85
96	3.6		
108			92
109		60.0	
128		157.0	
131			75
133			Death.

TABLE II.

Day of experiment.	Creatinine per 100 cc.	Urea N per 100 cc.	Blood pressure.
	mg.	*mg.*	
1			95
5			83
7			1st operation.
43			2nd operation.
63		27.0	
69		29.0	
109	2.2		
126	2.3		
147			90
211			Death.

Death Nov. 18. The kidney remnant showed a calculus in the pelvis and marked atrophy of the parenchyma. The anatomic evidence indicated death from renal insufficiency.

3. Jan. 13, 1923. Left kidney removed. Feb. 17, about 40 per cent of right kidney infarcted. Delivered of young Apr. 20. Death Apr. 23. There was a little increase of creatinine and urea nitrogen (Table III). The blood pressure was not notably affected. The kidney remnant was very large, weighing 11.9 gm. The portions not included in the infarcts showed moderate atrophy microscopically.

4. Mar. 22, 1922. 2 gm. of left kidney removed. Apr. 8, right kidney removed; weight 7.3 gm. The duration of this experiment was about 16 months. The animal escaped on July 7, 1923, and consequently the final condition of the remnant is unknown. There was a definite retention of creatinine and urea

TABLE III.

Day of experiment.	Creatinine per 100 cc.	Urea N per 100 cc.	Blood pressure.
	mg.	*mg.*	
1			75
34			84
158			1st operation.
179	2.0	20.1	
184			76
193			2nd operation.
214			90
219	2.1	26.1	
245			87
251	2.4	27.1	
258			Death.

TABLE IV.

Day of experiment.	Creatinine per 100 cc.	Urea N per 100 cc.	Blood pressure.
	mg.	*mg.*	
1	1.0		
4			1st operation.
8			72
21			2nd operation.
30	2.4	17.0	
35			87
40			83
44	2.6	22.4	
51	2.6		
53			78
60			81
68	1.8	17.3	
109			83
125		31.3	
161	2.1		
181			87
208			92
245			85
247			98
270		21.0	
280			84
304			77
307	1.8		
391	2.7	28.0	
398			74
399	2.0	20.0	
415	3.2	43.9	
437	3.0	35.0	
444			79

nitrogen during the first months (Table IV). The rabbit was fed a high protein diet from Apr. 11 to 15, 1923, from May 3 to 9, and from May 24 to 31. This presumably caused the retention of metabolites on the corresponding dates. A normal control rabbit, fed the same diet, showed no retention of metabolites.

The blood pressure was a little high on a single occasion in November but was otherwise within normal limits. The fundi were negative on May 18, Aug. 8, Dec. 12, 1922, and on May 7, 1923. The range in specific gravity was from 4 to 8 points.

TABLE V.

Day of experiment.	Creatinine per 100 cc.	Urea N per 100 cc.	Blood pressure.
	mg.	*mg.*	
1			81
18	1.6		
102			80
104			80
126		18.9	
137			88
157			1st operation.
177	2.1	9.7	
183			81
206			2nd operation.
213			86
218	2.9	38.0	
236			87
244			80
250	2.3	21.0	
257			73
258	3.3	67.0	
271			61
273	2.8	19.0	
279	1.9	14.0	
289			71
309	1.7	28.0	
359			68
365			83
399	1.5	17.5	
415			74
420			76

TABLE VI.

Day of experiment.	Creatinine per 100 cc.	Urea N per 100 cc.	Blood pressure.
	mg.	*mg.*	
1			57
3			1st operation.
7		11.4	
13	1.6	7.0	
17			2nd operation.
28			88
29	2.3	18.2	
34			88
36	2.5		
43	2.0	26.6	86
120		31.2	
133	2.0	23.6	
149			70.3
155			64
156			74

5. Jan. 13, 1923. 2.3 gm. of the left kidney removed. Mar. 3, right kidney removed; weight 8.3 gm. There was a moderate retention of metabolites following the second operation (Table V). Apr. 23, the animal developed a severe pyelonephritis with pus, blood cells, and casts in the urine and a marked retention in the blood. Later, when the rabbit recovered, the findings, as regards urea

nitrogen and creatinine in the blood, became normal and remained so except for the rise on June 14, following high protein feeding. The blood pressure remained normal throughout. The fundi were normal in appearance on Dec. 12, May 7, and Oct. 3. The rabbit is still alive.

6. May 5, 1923. 50 per cent of the left kidney infarcted. May 19, right kidney removed; weight 11.7 gm. The specific gravity showed a range of variation amounting to 20 points before operation and one of only 4 points after operation. There were slight rises in the urea nitrogen and creatinine, but the blood pressure remained normal (Table VI).

7. May 5, 1923. 50 per cent of left kidney infarcted. June 8, right kidney removed; weight 8.2 gm. Oct. 3, fundi normal. Aug. 30, slight retention of metabolites. The blood pressure was normal (Table VII).

TABLE VII.

Day of experiment.	Creatinine per 100 cc.	Urea N per 100 cc.	Blood pressure.
	mg.	*mg.*	
1			1st operation.
4		10.7	
34			2nd operation.
40			87
90			71
117	3.5	36.0	
130	1.7	21.7	
151			71

TABLE VIII.

Day of experiment.	Creatinine per 100 cc.	Urea N per 100 cc.	Blood pressure.
	mg.	*mg.*	
1			54
2			1st operation.
16			80
28	1.5	14.0	
30			2nd operation.
46	1.5	19.6	
48			64
53	1.9	18.9	83
54			73
58			64
59	1.76	30.0	

8. Aug. 11, 1923. 50 per cent of the left kidney infarcted. Sept. 8, right kidney removed; weight 8.3 gm. Oct. 3, fundi normal. Both the blood metabolites and the blood pressure remained within normal limits (Table VIII).

9. Aug. 11, 1923. 50 per cent of the left kidney infarcted. Sept. 8, right kidney removed; weight 7.1 gm. Oct. 3, fundi normal. Creatinine and urea nitrogen not notably increased and the blood pressure remained normal (Table IX).

10. Sept. 22, 1923. 2.5 gm. of the left kidney removed. Sept. 29, right kidney removed; weight 8.3 gm. Oct. 3, fundi negative. The blood metabolites showed slightly increased values. The pressure apparently went up following operation and remained above the preoperative readings (Table X).

11. Sept. 22, 1923. 2.3 gm. of the left kidney removed. Sept. 29, right kidney removed; weight 8.3 gm. Oct. 3, fundi negative. The blood metabolites underwent no significant rise excepting one of 2.4 mg. of creatinine on Oct. 3. The blood pressure apparently fell (Table XI).

TABLE IX.

Day of experiment.	Creatinine per 100 cc.	Urea N per 100 cc.	Blood pressure.
	mg.	*mg.*	
1			73
2			1st operation.
15			71
28	1.4	9.1	
30			2nd operation.
48	1.7	27.3	
50			88
55	1.7	19.6	87
56			84
61	1.9	26.0	

TABLE X.

Day of experiment.	Creatinine per 100 cc.	Urea N per 100 cc.	Blood pressure.
	mg.	*mg.*	
1			90
2			1st operation.
6	1.5	17.5	
8			88
9			2nd operation.
14	2.5	28.7	97
15			96
19			107
20	2.0	17.6	
48			98
50	2.0	35.7	

TABLE XI.

Day of experiment.	Creatinine per 100 cc.	Urea N per 100 cc.	Blood pressure.
	mg.	*mg.*	
1			87
2			1st operation.
6	1.5	11.9	
8			84
9			2nd operation.
14	2.4	21.0	73
15			68
20	2.1	21.4	69
48			82
50	2.0	33.6	

DISCUSSION.

The amount of kidney parenchyma removed or destroyed in these experiments was roughly 70 per cent. Two rabbits died of renal insufficiency (No. 1 from extension of scar and No. 2 from obstruc-

tion caused by a calculus). No. 3 had moderate renal insufficiency and showed moderate atrophy of the tubules at autopsy but may have died from a complicating condition following the delivery of young, rather than from the failure of renal function. In Nos. 4 to 11 (all still living) the renal function is apparently improving. This is presumably due to hypertrophy of the kidney remnant, which is known to occur.

In normal rabbits on ordinary diet, creatinine was found to average 1.6 mg. and urea nitrogen 19 mg. The high normal figures were 2.1 mg. and about 25 mg., respectively. Accepting these figures as normal, a definite retention of metabolites was present in all except Nos. 8, 9, and 11.

This retention occurred soon after the second operation but was followed later, except in the first three which died, by indications of improvement of renal function. The high creatinine and urea nitrogen during the latter part of Experiment 4 were brought about presumably by high protein feeding in the presence of a very limited renal function. The normal control showed no such rise from high protein feeding.

The average normal systolic blood pressure in the central artery of the rabbit's ear is about 78 mm. of mercury (600 readings on twenty-five normal rabbits). The usual limits are 75 to 88. The pressure is high when the animal is frightened and low when the ear is cold (5). Such conditions must be avoided. The tables show that the variations in the blood pressure in the experimental animals are practically all within normal limits except in the case of No. 10. The average pressure in the series before any operation was 78.2 mm. and after the second operation the average was 80.1 mm. These experiments indicate, therefore, that a marked reduction of kidney substance in the rabbit does not result in hypertension. By inference then, the atrophy of renal tissue in chronic glomerulonephritis is not the cause of the accompanying hypertension.

SUMMARY.

The removal or destruction of about 70 per cent of the kidney tissue in rabbits does not produce hypertension even when prolonged renal insufficiency results.

I gratefully acknowledge my indebtedness to Miss Florence Madsen, Minneapolis General Hospital, W. W. Swanson, University of Minnesota, and Mr. F. Rytz, St. Luke's Hospital of Duluth for aid in the blood chemistry determinations. I am also indebted to the authorities of St. Luke's Hospital for the use of laboratories in which to carry out this work, and to Dr. E. T. Bell, University of Minnesota, for advice and encouragement.

BIBLIOGRAPHY.

1. Jawein, G., *Berl. klin. Woch.*, 1920, lvii, 869.
2. Pilcher, J. D., *J. Biol. Chem.*, 1913, xiv, 389.
3. Pässler, H., and Heinike, *Verhandl. deutsch. path. Ges.*, 1905, ix, 99.
4. Janeway, T. C., *Proc. Soc. Exp. Biol. and Med.*, 1908–09, vi, 109.
5. Anderson, H. C., *Proc. Soc. Exp. Biol. and Med.*, 1922–23, xx, 295.
6. Bradford, J. R., *J. Physiol.*, 1898–99, xxiii, 415.
7. Folin, C., and Wu, H., *J. Biol. Chem.*, 1919, xxxviii, 81.

SURFACE TENSION OF SERUM.

VIII. Further Evidence Indicating the Existence of a Superficial Polarized Layer of Molecules at Certain Dilutions.

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Plate 37.

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In order to account for a certain number of phenomena described and studied in Papers I to VI,[1] it has been assumed that a monomolecular layer of oriented molecules exists at a given dilution of the serum; namely, 1:10,000. The term monomolecular was used for convenience, but should not be taken in its definite chemical sense. The term monomicellar could have been used, but as micellæ have never been looked upon as having a definite architecture, and as their structure is still subject to controversy, it was thought better to use the word molecule provisionally. Consequently, by monomolecular film, or layer, we mean a layer of organized molecules or of organized groups of molecules which can be bound together by molecular forces.

If such a film really exists at a dilution of 1:10,000, it seems that the rate of evaporation, in a watch-glass, should be different from that at other concentrations, since the homogeneity of the film should prevent water molecules from escaping from the surface as easily. This solution should, therefore, evaporate more slowly. At higher concentrations, the serum molecules are disposed in thicker layers. As they are presumably piled up and not oriented, the small water molecules can pass between the large agglomerations of micellæ and evaporate more rapidly. The same thing should happen at high

[1] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 575, 707; xxxvi, 115, 547; 1923, xxxvii, 659; xxxviii, 87.

dilution, when the number of serum molecules is no longer sufficient to cover the surface of the water. A very crude comparison will perhaps make this clear.

Suppose three square tanks, whose bases measure 20 by 20 cm., are filled with water. Into the first tank, 800 or 1,000 small wax prisms, 1 by 1 by 2 cm., are thrown. They will float and pile up in irregular layers and will not cover the surface of the water evenly, but in a rather chaotic way. Through the numerous channels left between them, the air will come in contact with the water, and evaporation will take place. Such an arrangement may be thought to correspond to a high serum concentration, that from 10^{-1} to 10^{-3}. In the second tank, only 100 prisms are placed. They will float horizontally or even vertically, if four or more stick together, but relatively large areas of water will be free from them as the number is not large enough to cover the whole surface. This condition is analogous to that at low serum concentrations, from 10^{-5} up. In the third tank, exactly 400 prisms are placed vertically, side by side, so as to cover the surface of the water smoothly and completely. There will be no contact between the water and the air, except through crannies, and evaporation will be very slow. The surface covering corresponds to the oriented layer existing at the optimum dilution of serum for our vessels; namely, 10^{-4}.

The results of the experiments made to test this assumption are given in Tables I to IV, which express the diameter of the surface of the liquid in watch-glasses, towards the end of evaporation. Between 4 and 6 hours were required to reduce the original diameter of 40 mm. to those recorded in the first line of Tables I to IV (Text-fig. 1).

In another experiment (Table V), after 5 hours and 20 minutes, evaporation was progressing very slowly. For this reason, a fan was turned on the watch-glasses, and a very rapid evaporation followed.

The solutions of Sera 226, 227, 228, and 229 (Tables I to IV) were kept in test-tubes at room temperature, and the next day exposed to evaporation exactly as before. This time, it seemed that the slowest evaporation tended to shift to the 1:1,000 dilution; the difference was not so marked as in previous experiments between the diameters of the 1:10,000 solution and the 1:1,000. The reason may be that large aggregates are formed overnight, the molecules associate in some way, and the 1:1,000 solution, despite its higher concentration, is covered with a more homogeneous film than the 1:10,000 solution. This view, though purely hypothetical, appears

to be supported by the fact that, when the solutions are prepared from very fresh serum, there is a tendency for a shift to occur in the

TABLE I.

Serum 226.

Diameter of Serum Solutions (Rabbit) Evaporating in Watch-Glasses.

Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Diameter in mm. at 5.41 p.m., after 6 hrs. evaporation	26	27	27	30	27	26
After 30 min	20	20	23	26.5	23	22
" 1 hr.	0	0	6	14	0	0

TABLE II.

Serum 227.

Diameter of Serum Solutions (Rabbit) Evaporating in Watch-Glasses.

Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Diameter in mm. at 5.42 p.m.	18	23	21	21	20	20
After 50 min.	0	5	8	12	0	0

TABLE III.

Serum 228.

Diameter of Serum Solutions (Rabbit) Evaporating in Watch-Glasses.

Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Diameter in mm. at 5.45 p.m.	18	18	12	22	13	18
After 30 min.	0	0	0	15	0	0

TABLE IV.

Serum 229.

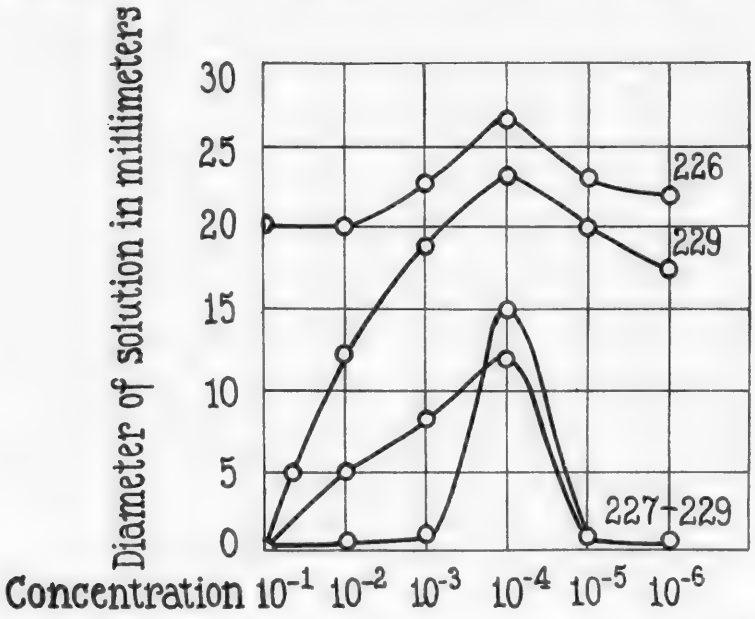
Diameter of Serum Solutions (Rabbit) Evaporating in Watch-Glasses.

Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Diameter in mm. at 5.50 p.m.	0	20	23	26	25	23
After 30 min.	0	12	19	23	22	18
" 40 "	0	0	6	18	6	0

evaporation rate towards the 1:100,000 solution, which evaporates more slowly. However, in the great majority of cases, it can be said

that a markedly slower evaporation rate is to be noted at a dilution of 1:10,000. It is always slower between 1:1,000 and 1:100,000 than at other dilutions.

An attempt was made to improve the technique employed by spreading only 0.5 cc. of solution on a piece of plate glass, over a



TEXT-FIG. 1. Diameter of serum solutions in watch-glasses after a few hours evaporation, showing that the solutions to 1:10,000 evaporated more slowly than the others (*cf.* Tables I to IV).

circle 50 mm. in diameter. The size of the circle and the quantity of liquid were measured as accurately as possible. Tables VIII to XII give the time required for 0.5 cc. of solution to evaporate completely. The zero was arbitrarily taken as the moment one solution, whichever it was, dried out completely, and dilutions were arranged according to their greater tendency to evaporate.

It will be seen that the ratio, $\frac{\text{Surface}}{\text{Volume}}$, is not the same in the case of 0.5 cc. spread over an area of 18.6 sq. cm. as in our standard watch-

TABLE V.

Serum 237.

Diameter of Serum Solutions (Rabbit) Evaporating in Watch-Glasses.

Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Diameter in mm. at 2.20 p.m.	8	11	4	20	20	19
After 5 min.	0	3	0	16	14	10
" 10 "	0	0	0	13	8	0
" 15 "	0	0	0	11	0	0
" 20 "	0	0	0	10	0	0
" 25 "	0	0	0	6	0	0
" 30 "	0	0	0	0	0	0

TABLE VI.

Serum 236. (*Fan Blowing.*)

Diameter of Serum Solutions (Rabbit) Evaporating in Watch-Glasses.

Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Diameter in mm. at 3.00 p.m.	7	4	0	8	7	0
After 5 min.	4	0	0	5	0	0
" 7 "	0	0	0	4	0	0
" 12 "	0	0	0	0	0	0

TABLE VII.

Serum 235. (*Fan Blowing.*)

Diameter of Serum Solutions (Rabbit) Evaporating in Watch-Glasses.

Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Diameter in mm. at 4.39 p.m.	14	10	14	20	20	19
After 6 min.	11	0	8	19	19	18
" 11 "	10	0	0	19	19	18
" 21 "	6	0	0	18	18	10
" 24 "	0	0	0	18	Became very irregular in shape; did not wet glass.	0
" 31 "	0	0	0	12		0
" 45 "	0	0	0	8		0
" 51 "	0	0	0	0		0

glasses. In the watch-glasses, it is roughly 12; in the present case, 74. The ratio of these two figures is approximately 6.16. Consequently, the concentration should be about six times greater in order to produce the monomolecular film and, according to the hypothesis

TABLE VIII.

Serum 230.
Evaporation of Serum Solutions (Rabbit).

Dilution	10^{-5}	10^{-6}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Time in min.	0	1	2	12	14	16

The figures indicate the number of minutes which were required by each solution to dry, the zero time being taken when one of them, no matter which, dried first.

TABLE IX.

Serum 231.
Evaporation of Serum Solutions (Rabbit).

Dilution	10^{-6}	10^{-5}	10^{-1}	10^{-3}	10^{-2}	10^{-4}
Time in min.	0	2	5	12	12 min., 30 sec.	18 min., 15 sec.

The figures indicate the number of minutes which were required by each solution to dry, the zero time being taken when one of them, no matter which, dried first.

TABLE X.

Serum 237.
Evaporation of Serum Solutions (Rabbit).

Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Time in min.	0	1	2	9	Solutions did not wet the glass evenly.	

The figures indicate the number of minutes which were required by each solution to dry, the zero time being taken when one of them, no matter which, dried first.

now under consideration, the 10^{-4} solution should evaporate more rapidly than the 1:1,650 solution. However, experiments showed that there was little difference between the rate of evaporation of these two concentrations. The reason for this is not quite plain.

Perhaps, the extreme thinness of the liquid may have affected the normal formation of the monomolecular layer.

These experiments, although apparently quite simple, are not easy to carry out on account of the difficulty in obtaining a large piece of plate glass which will wet thoroughly and evenly, and in leveling it so as to obtain a perfectly even evaporation of the whole area, without any irregular thinning of the solutions. It was only

TABLE XI.

Serum 236.
Evaporation of Serum Solutions (Rabbit).

Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Time in min	0	26	31	50	Solutions did not wet the glass evenly.	

The figures indicate the number of minutes which were required by each solution to dry, the zero time being taken when one of them, no matter which, dried first.

TABLE XII.

Serum 236.
Evaporation of Serum Solutions (Rabbit).

Dilution	1:1,000	1:20,000	(10^{-4}) 1:10,000	1:1,650
Time in min	0	12	15	16

The figures indicate the number of minutes which were required by each solution to dry, the zero time being taken when one of them, no matter which, dried first.

by frequent washing in a boiling cleaning fluid and almost immediate utilization of the plates that it was possible to succeed. Of course, the higher dilutions, on account of their high surface tension, were the most difficult to spread. In order to prevent the spreading of the lower dilutions outside the limits of the 50 mm. diameter circle, a very thin circumference was traced on the glass with a wax pencil. Although almost invisible, this line was an absolute barrier to the solutions. Fig. 1 shows the result of a successful experiment, and Fig. 2 the result when the glass was not level, which prevented even evaporation.

Thus, in all cases, it was found that evaporation was decidedly slower at a concentration near 1:10,000. This confirms our assumption, and it seems difficult otherwise to account for the phenomenon.

CONCLUSIONS.

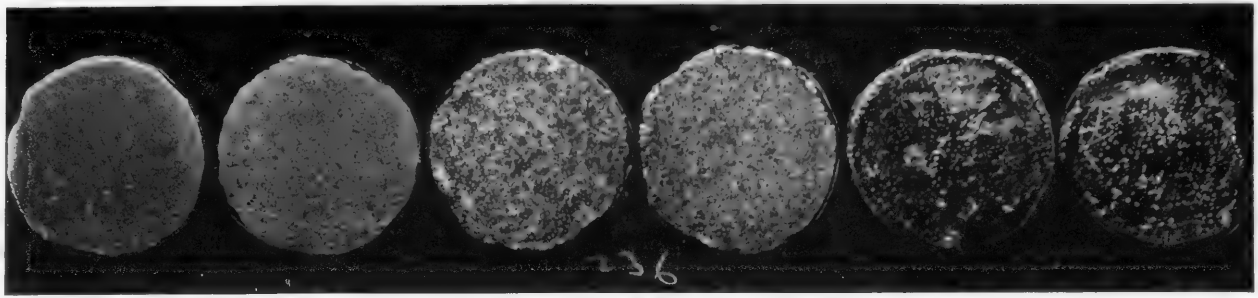
The assumption has been made in preceding papers that, at a given concentration of 1:10,000, in the vessels used in our experiments, a monomolecular oriented layer was formed. Such a layer might be supposed to prevent the free escape of water molecules from the surface into the air, at least to a certain extent.

In order to check this assumption, the rate of evaporation of solutions of serum at different concentrations was measured. It was found that, under the conditions of the experiments, in a progression of dilutions from 10^{-1} to 10^{-6}, the slowest evaporation took place at a concentration of 1:10,000. In a few cases (less than 20 per cent), evaporation was slower at a different concentration, but always within the same range (between 10^{-3} and 10^{-5}), not far from 10^{-4}.

EXPLANATION OF PLATE 37.

FIG. 1. The leveling of the plate was good, and the solutions evaporated evenly, drying everywhere at about the same moment save at a small spot near the center of the disk, where there was some delay.

FIG. 2. The leveling was bad and the solutions started drying on one part of the circumference, while the liquid accumulated on the opposite side.



Concentration of serum in saline at 0.9 per cent..... 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6}

FIG. 1.

Concentration of serum in saline at 0.9 per cent..... 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6}

FIG. 2.

THE INFLUENCE OF RESISTANCE ON THE CHARACTER OF DISEASE IN EXPERIMENTAL RESPIRATORY INFECTION.

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It has become well recognized that organisms of considerable virulence may be introduced into the uninjured respiratory tract without producing recognizable disease. Arloing, Cornevin, and Thomas[1] called attention to the fact that injection of fluids containing the bacilli of symptomatic anthrax into the trachea of calves resulted in immunity but failed to produce disease, provided there was no leakage of material into the peritracheal tissues. Snel's[2] experiments with the anthrax bacillus gave similar results. He injected into the trachea of guinea pigs the vegetative and spore forms of the anthrax bacillus. When the organisms were introduced by means of a catheter passed through the larynx the animals remained well. Guinea pigs injected by means of a hypodermic needle thrust between the rings of the trachea died of septicemia. Snel pointed out that a locus occurred about the site of injection, from which the organisms entered the circulation.

A number of workers have succeeded in producing pneumonia in animals by intratracheal injection of various organisms. In most instances it has been necessary to force considerable amounts of culture well down into the trachea before positive results have been obtained. Wadsworth[3] was able under certain conditions to produce pneumonia in rabbits by the injection of 1 cc. of pneumococci between

[1] Arloing, Cornevin, and Thomas, cited by Kitt, T., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen. Jena, 2nd edition, 1912, iv, 819.

[2] Snel, J. J., *Z. Hyg. u. Infectionskrankh.*, 1902, xl, 103.

[3] Wadsworth, A., *Am. J. Med. Sc.*, 1904, cxxvii, 851.

the tracheal rings. It is of interest that in his first experiments the rabbits either developed septicemia without lung involvement or remained well. Kline and Winternitz[4] produced pneumonia in rabbits with the pneumococcus by intratracheal injection of large amounts of culture. They pointed out that the production of pneumonia depended on the introduction of the organisms into the alveoli. In order to accomplish this, it was necessary to insert a catheter as deeply into the bronchus as possible. The liquid culture was injected with considerable force.

During the past year the writer[5] has developed a simple and rapid method for introducing fluids into the trachea. A properly bent metal tube with a rounded end may be inserted by way of the mouth through the larynx. By attaching a syringe varying amounts of fluid may be injected. Aside from the extreme simplicity of the procedure, no apparent injury is produced. In addition, the rabbit is placed in the natural position so that it is necessary to exert only a slight pressure on the piston of the syringe to insure that the material reaches the lungs.

The pathogenicity of a number of organisms introduced in this manner has been tested. Members of the Pasteurella group associated with pneumonias in animals, such as the swine-plague bacillus, certain of the hemorrhagic septicemia organisms of bovine origin, and *Bacillus lepisepticus*, possess considerable pathogenicity when introduced beneath the skin or into the circulation. They rarely produce disease when introduced into the uninjured trachea. The animals usually develop a temperature reaction for a few hours, but fail to manifest further symptoms. The recent experiments of Stillman[6] are of considerable interest in this connection. He exposed mice to an atmosphere containing cultures of various organisms in the form of a fine mist. He was able to show that the organisms reached the lower respiratory tract. The pneumococcus usually disappeared from the lungs within a few hours. Hemolytic streptococci persisted for longer periods and gave rise to septicemia in many instances.

[4] Kline, B. S., and Winternitz, M. C., *J. Exp. Med.*, 1915, xxi, 304.
[5] Jones, F. S., *J. Exp. Med.*, 1923, xxxvii,789.
[6] Stillman, E. G., *J. Exp. Med.*, 1923, xxxviii, 117.

Organisms of the chicken-cholera group are usually extremely virulent for rabbits. The writer is in possession of a culture of *Bacillus avisepticus* which when introduced into the trachea in small quantities gives rise to a fatal septicemia. 1/100 cc. of a 24 hour bouillon culture suspended in 1 cc. of sterile bouillon injected into the trachea always gives rise to septicemia which terminates fatally in a little over 24 hours. With this amount of culture the average length of time between inoculation and death has been 28 hours, the minimum 17 hours and the maximum 37 hours.

It seemed possible that under certain conditions such an organism, which is capable of passing through the walls of the lower respiratory tract, might be made to localize within the lung. Ample evidence exists that other members of the hemorrhagic septicemia groups, *Bacillus lepisepticus*, *Bacillus bovisepticus*, and others, are associated with pneumonia in their respective hosts. In addition, it might be possible by experimentation to throw some light on the general problem of pneumonia. With these points in view a number of experiments were undertaken.

EXPERIMENTAL.

Rabbits from a stock which is relatively free of spontaneous respiratory disease were used throughout the experiments. Those of approximately the same age, breed, and weight (2,000 gm.) were employed. Intratracheal injections were made with the curved metal tube. Sufficient ether was given to insure complete relaxation. 1/100 cc. of a 24 hour bouillon culture suspended in 1 cc. of sterile bouillon was always used as the infecting dose. It has been stated that this dose always proved fatal; the initial experiments dealing with this point will be omitted, since protocols given later include animals which received only the infecting dose.

Smith and Moore[7] were able to vary by vaccination the type of disease produced by injection of highly virulent cultures of the swine-plague bacillus. Unvaccinated control rabbits rapidly succumbed to septicemia after subcutaneous inoculation with living cultures. Rabbits that had received four injections of killed culture were subsequently inoculated with live culture. They developed large

[7] Smith, T., and Moore, V. A., *U. S. Dept. Agric., Bureau of Animal Industry, 8th and 9th Ann. Rep.*, 1891–92, 45.

TABLE I.

The Protocols of Rabbits Immunized with Killed Culture by Various Routes and Subsequently Inoculated with Living Culture.

Rabbit 1. Vaccinated intraperitoneally.	Rabbit 2. Vaccinated intratracheally.
1922	*1922*
Dec. 28. 1 cc. of vaccine.	Dec. 27. 1 cc. of vaccine.
1923	*1923*
Jan. 2. 1 " " "	Jan. 2. 1 " " "
" 6. 1.5 " " "	" 6. 1.5 " " "
" 15. Agglutinins +++ 1:200.	" 15. Agglutinins + 1:100.
" 16. 1/100 cc. of living culture intratracheally.	" 16. 1/100 cc. of living culture intratracheally.
" 17. Blood culture negative. Animal normal.	" 17. Blood culture negative. Animal normal.
" 23. Agglutinins ++ 1:500.	" 23. Agglutinins + 1:500.
1/10 cc. of living culture intratracheally.	1/10 cc. of living culture intratracheally.
" 31. 1 " " " " "	" 31. 1/2 " " " " "
Feb. 7. 1/100 " " " " subcutaneously.	Agglutinins + 1:500.
" 9. Abscess.	Feb. 7. 1/100 cc. of living culture subcutaneously.
Animal recovered.	" 9. Abscess.
	Animal recovered.

Rabbit 3. Vaccinated subcutaneously.	Rabbit 4. Vaccinated intraperitoneally.
1923	*1923*
Jan. 29. 1 cc. of vaccine.	Jan. 29. 1 cc. of vaccine.
Feb. 3. 1 " " "	Feb. 3. 1.5 " " "
" 12. Agglutinins complete 1:50.	" 12. Agglutinins ++++ 1:100.
1/100 cc. of living culture intratracheally.	1/100 cc. of living culture subcutaneously.
Remained well.	" 13. Animal very sick.
	" 14. Large abscess. Animal very sick. Later the abscess ulcerated. Animal finally recovered.

Rabbit 5.
Unvaccinated control.*

1923		
Jan.	16.	1/100 cc. of living culture intratracheally.
"	17.	Blood culture positive.
		Died of septicemia 29 hrs. after inoculation.

* Normal rabbits injected subcutaneously with 1/100 cc. of bouillon culture succumbed within 48 hours.

abscesses about the site of inoculation and survived much longer than the controls.

The first phase of the problem consisted in establishing whether rabbits could be protected against the experimental respiratory infection with this strain of *Bacillus avisepticus*. In addition, it was essential to show whether the general infection could be limited to a local process about the site of inoculation. With these points in view two lots of rabbits were tested after various treatments. Inasmuch as the series are small and the results similar, a brief record of all rabbits is given in Table I.

The vaccines used throughout the experiments were prepared by suspending the surface growth from 24 hour agar slant cultures in small quantities of salt solution to which 0.5 per cent phenol had been added. The heavy suspensions were heated for 1 hour at 58–60°C. Before use sufficient sterile salt solution was added to reduce the turbidity to 2.5 with the Gates apparatus.

It is evident from the data given in Table I that a more or less complete immunity to a subsequent intratracheal infection may result from intraperitoneal, intratracheal, or subcutaneous vaccination with killed cultures. Although it is not known how little vaccine is required to produce complete resistance, in the case of Rabbit 3, 2 cc. of vaccine administered beneath the skin completely protected. Rabbits 1 and 2 were able to tolerate many times the lethal dose, provided however the living culture was introduced into the respiratory tract. Such was not the case when the organisms were injected beneath the skin, since the bacteria multiplied in the subcutaneous tissue and produced an abscess. It is evident that immunity to respiratory infection may be readily induced provided the integument is not seriously injured. Here the natural mechanism is not interfered with and the added resistance stimulated by the vaccine soon enables the individual to overcome the infection.

The experiment also parallels Smith and Moore's findings with the swine-plague bacillus that the type of disease could be changed from a rapidly fatal septicemia to a process local in character which spread by continuity.

As a basis for further experiments three points had been established: (1) 1/100 cc. of a 24 hour bouillon culture when introduced into the trachea of normal rabbits will always produce a rapidly fatal septi-

cemia. (2) Infection through the respiratory tract may be completely prevented by previously administering small doses of vaccine subcutaneously, intraperitoneally, or intratracheally. (3) By stimulating resistance the process may be changed from a septicemia to a local process beneath the skin.

On the basis of these facts, it was postulated that a partial protection or resistance somewhat greater than that possessed by the normal individual, but considerably less than that of complete protection, would, on intratracheal injection, tend to produce a localization within the lung. The later experiments resolved themselves into attempts, by raising the resistance slightly above the normal by means of vaccination with killed cultures, to change the disease from an acutely fatal septicemia to a less acute pneumonia.

Cecil and Steffen[8] were able to show that intratracheal injections of killed cultures rendered monkeys insusceptible to later intratracheal injections of living pneumococci. Among other experiments they attempted to produce immunity by spraying the vaccine into the pharynx. They were unable to establish immunity by this method. Such a method seemed desirable for experimenal purposes. Attempts were made to increase the resistance of rabbits to subsequent intratracheal injection by spraying killed cultures into the nostrils and throat.

Experiment 2.—As in the preceding experiments, rabbits averaging 2,000 gm. in weight were employed. Vaccine prepared in the usual manner but of greater turbidity (1.5) was sprayed into the nostrils of No. 6 and into the pharynx of No. 7. No. 8 served as a control. The results are given in Table II.

The experiment was repeated with the same results. It was not possible by means of sprays of vaccine to increase the resistance appreciably. The blood failed to show agglutinins throughout the experiment. Temperature reactions or leucocytosis did not occur following the administration of vaccine. Apparently insufficient antigen is absorbed to afford measurable protection against the subsequent intratracheal infection.

In the third series of experiments it was decided to inject subcutaneously a number of rabbits with 1 cc. of vaccine (turbidity 2.5)

[8] Cecil, R. L., and Steffen, G. I., *Pub. Health Rep., U. S. P. H.*, 1922, xxxvii, 2735.

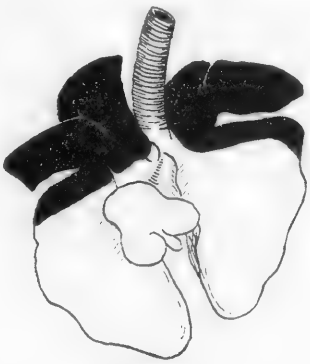
TABLE II.

Attempts to Increase the Resistance of Rabbits against Intratracheal Injection by Spraying Killed Cultures on the Mucous Membrane of the Nostrils and Pharynx.

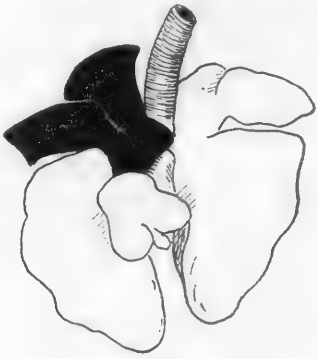
Rabbit 6. Vaccine sprayed into the nostrils.	Rabbit 7. Vaccine sprayed into the pharynx.	Rabbit 8 Control.
1923	*1923*	
Mar. 26. Nasal mucosa sprayed with vaccine.	Mar. 26. Throat sprayed with vaccine.	
" 27. " " " " "	" 27. " " " "	
" 28. " " " " "	" 28. " " " "	
Apr. 2. " " " " "	Apr. 2. " " " "	
" 3. " " " " "	" 3. " " " "	
" 4. " " " " "	" 4. " " " "	
" 11. 1/100 cc. of living culture intratracheally.	" 11. 1/100 cc. of living culture intratracheally.	Apr. 11. 1/100 cc. of living culture intratracheally.
" 12. Died (36 hrs. after injection). Septicemia.	" 12. Died (22 hrs. after injection). Septicemia. Fibrinous pericarditis. Pleuritis with effusion.	" 12. Died (23 hrs. after injection). Septicemia.

and after definite intervals to inject into the trachea 1/100 cc. of living culture.

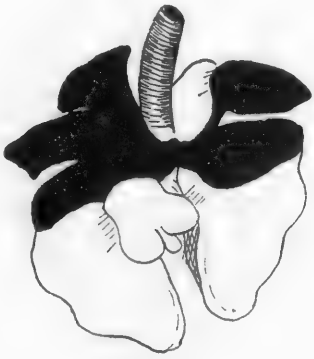
In one experiment three rabbits were vaccinated and 2, 4, and 7 days later injected intratracheally with 1/100 cc. of living culture. An unvaccinated rabbit



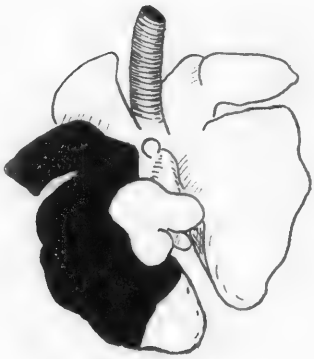
TEXT-FIG. 1.



TEXT-FIG. 2.



TEXT-FIG. 3.



TEXT-FIG. 4.

Consolidation is shown in black.

TEXT-FIG. 1. The ventral aspect of the lungs of Rabbit 9.
TEXT-FIG. 2. The ventral aspect of the lungs of Rabbit 10.
TEXT-FIG. 3. The ventral aspect of the lungs of Rabbit 12.
TEXT-FIG. 4. The ventral aspect of the lungs of Rabbit 16.

served as control. In another experiment three rabbits were vaccinated and infected 3, 5, and 6 days later. A fourth rabbit served as a control. Inasmuch

as some of the clinical evidence is of considerable interest an outline of temperature reactions, the results of blood cultures, and in certain instances the findings after pleural puncture are appended.

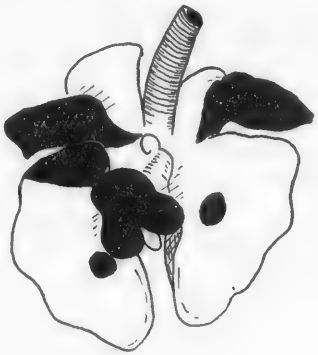
Blood cultures were always made from the peripheral circulation. 1 cc. of blood may be readily drawn from the ear artery or marginal vein with a 2 cc. Luer syringe, provided the ear is warmed to increase the peripheral circulation. The median artery is better for the purpose, since the pressure may be sufficient to fill the syringe. The advantage of the use of the vessels of the peripheral circulation over the cardiac method for blood culture in diseases involving the thoracic organs is obvious, especially when the organisms are numerous in the exudates covering the pericardium and pleura.

Rabbit 9.—(Text-figs. 1 and 7, *a*.) Vaccinated 2 days before infection. Septicemia developed during the first 24 hours. The rabbit died 56 hours after infection. Autopsy revealed severe pleuritis and pericarditis. The pleural cavity contained 5 cc. of serous fluid. The ventral and cephalic lobes and adjacent portions of the caudal lobes of both lungs were completely consolidated. The solid portions were deep red in color. The air-containing portions of the lung were congested and edematous. Microscopically the air cells of the involved lobe were filled with red cells, red cells and fibrin, and a few round cells. The vascular system was engorged. The epithelium of the air tubes was degenerated and often the more superficial layers had exfoliated.

Rabbit 10.—(Text-figs. 2 and 7, *b*.) Vaccinated 4 days before infection. Septicemia did not develop during the first 24 hours. The animal reacted severely and died 69 hours after the intratracheal injection. On autopsy 4 cc. of turbid exudate were found in the pleural cavity. There was a marked fibrinous pericarditis and pleuritis. The ventral and cephalic lobes of the right lung were much enlarged. On section they were firm and varied from reddish gray to red in color. The cephalic lobe of the left lung was shrunken and deep red. The other lobes were congested and edematous. Microscopically the process in the cephalic lobe was that of intense pleuritis. Leucocytes had invaded the pleura. Hemorrhages had occurred in portions of the sections beneath the pleura. In the center of the lobe many of the alveoli were filled with red cells. The vascular engorgement was intense. The ventral lobe manifested two well defined types of pneumonia rather sharply differentiated. In one portion the exudate which filled the alveoli was largely fibrinous; in the remainder of the lobe the air spaces were packed with leucocytes. Severe degeneration of the epithelium of the air tubes was constant in both sections.

Rabbit 11.—(Text-fig. 7, *c*.) Vaccinated 7 days before infection. The animal showed no particular reaction to the usual intratracheal dose of culture. 2 days after the first infection the rabbit was again injected with a larger amount of culture. A characteristic temperature reaction developed for a few hours after the second injection. It was of short duration. 8 days after the last injection the rabbit was killed. Autopsy failed to reveal abnormalities.

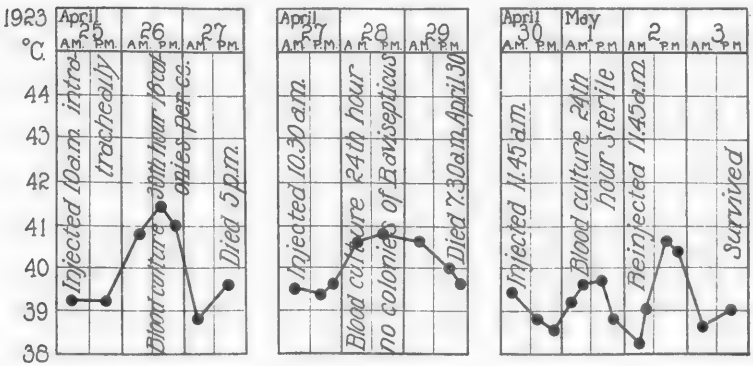
Rabbit 12.—(Text-figs. 3 and 8.) Vaccinated 3 days before infection. Septicemia was not manifested during the first 24 hours. The organisms were present in the blood after this time. The rabbit died 88 hours after infection. Postmortem examination revealed 6 cc. of turbid, serous exudate in the pleural cavity. The usual fibrinous pericarditis and pleuritis were encountered. Extensive



TEXT-FIG. 5.

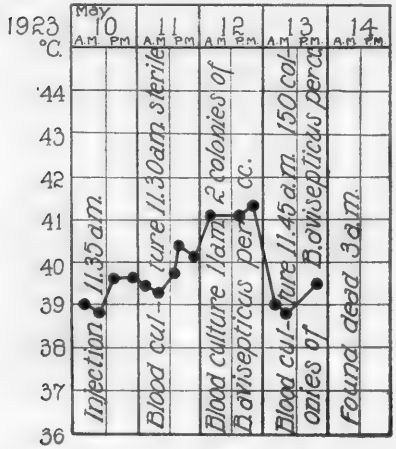
TEXT-FIG. 6.

TEXT-FIG. 5. The ventral aspect of the lungs of Rabbit 20.
TEXT-FIG. 6. The ventral aspect of the lungs of Rabbit 21.

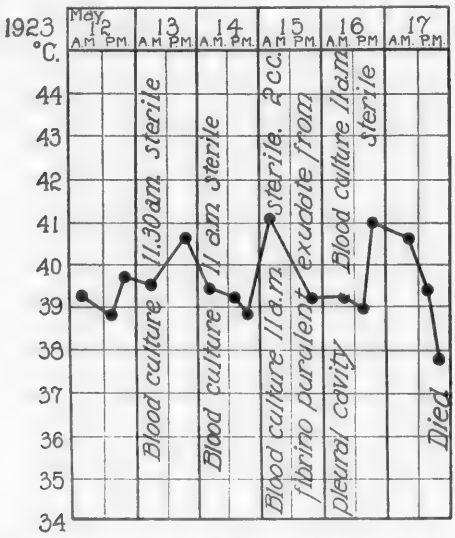


a *b* *c*

TEXT-FIG. 7, *a* to *c*. Temperature curves. (*a*) Rabbit 9, vaccinated 2 days before intratracheal injection. (*b*) Rabbit 10, vaccinated 4 days before intratracheal injection. (*c*) Rabbit 11, vaccinated 7 days before intratracheal injection.

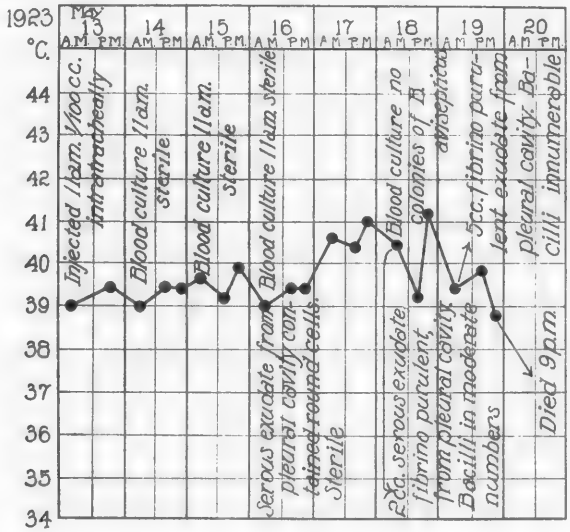


TEXT-FIG. 8. Temperature curve of Rabbit 12, vaccinated 3 days before intratracheal injection.



TEXT-FIG. 9. Temperature curve of Rabbit 13, vaccinated 5 days before intratracheal injection.

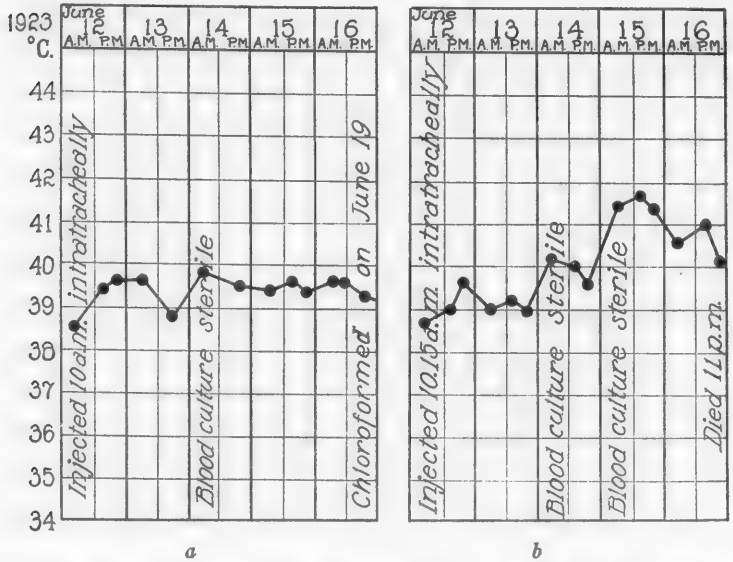
consolidations, varying in color from bright red to gray, of the more dependent portions of both lungs were observed. On the whole the gray type of consolidation was more pronounced in the left lung. Microscopically the character of the pneumonia varied in different lobes and within sections of the same lobes. The more dependent portions of the left ventral lobe revealed very little of the normal lung structure. The whole consisted of a mass of necrotic leucocytes, evidently the remains of the alveolar exudate. In other portions of the lobe the alveoli were filled with fibrin. Here the vascular engorgement was intense. In other lobes the air spaces were packed with red cells. All sections showed severe degeneration or necrosis of the epithelium of the air tubes. Often fibrinous exudate filled the lumen of the air tubes. In many instances the peribronchial blood vessels were engorged and the peribronchial lymph channels were occluded with fibrin.



TEXT-FIG. 10. Temperature curve of Rabbit 14, vaccinated 6 days before intratracheal injection.

Rabbit 13.—(Text-fig. 9.) Vaccinated 5 days before infection. The temperature reaction was irregular. The blood was sterile during the first 4 days of illness. Evidences of empyema were detected on the 3rd day when a serous exudate containing polymorphs and the bacilli was aspirated from the pleural cavity. The animal died 5½ days after inoculation. At autopsy, 5.5 cc. of purulent exudate were obtained from the chest cavity. A heavy, yellowish white, fibrinopurulent exudate was found over the pericardium, the entire right lung, and portions of the left lung. The exudate peeled off with gentle traction, leaving

a roughened surface. The cephalic lobe of the right lung was collapsed. The other lobes were congested and edematous. Microscopically the lesions were essentially those of purulent pleuritis. The endothelium was overlaid with leucocytes. Leucocytes had invaded the subpleural tissues. The alveoli immediately adjacent to the pleura were overrun with leucocytes. Elsewhere the air spaces contained only a little finely granular material or a few round cells. The mucosa of the bronchi and bronchioles revealed only slight degenerative changes. A moderate engorgement of the interalveolar capillaries was also observed.



a *b*

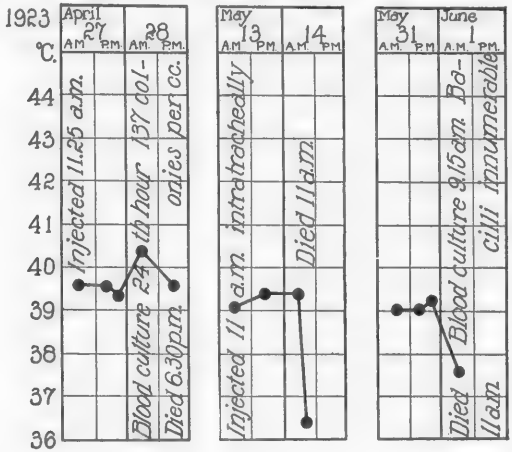
TEXT-FIG. 11, *a* and *b*. Temperature curves. (*a*) Rabbit 15. (*b*) Rabbit 16. Each received two injections of vaccine at 3 day intervals. Intratracheal injection 3 days after second vaccination.

Rabbit 14.—(Text-fig. 10.) Vaccinated 6 days before infection. The onset of symptoms was delayed. The first rise in temperature was noted on the 4th day. The blood remained free from *B. avisepticus* for 5 days. On the 5th and 6th days considerable amounts of purulent exudate were obtained by pleural puncture. The animal succumbed 6½ days after intratracheal injection. The autopsy findings were similar to those encountered in Rabbit 13. In addition there was a recent, deep red consolidation of the ventral lobe of the right lung. Microscopic examination showed a hemorrhage of considerable proportion in this lobe. Pleuritis was marked in all sections. Leucocytes had infiltrated the

pleura and adjoining portions of the lung. The organisms appeared in cloud-like masses in the pleura and subpleural structures. The alveoli and air tubes were not particularly involved.

Rabbit 15.—(Text-fig. 11, *a*.) Vaccinated twice at 3 day intervals. Injected intratracheally 3 days after second vaccination. The animal remained well. On three occasions the pleural cavity was tapped and a little clear, straw-colored fluid obtained. Inoculations of media from this liquid remained sterile. The animal was chloroformed 7 days after intratracheal injection. The lungs and chest cavity were normal.

Rabbit 16.—(Text-figs. 4 and 11, *b*.) Treated the same as Rabbit 15. Reacted on the 2nd day after infection. The blood remained sterile during the first 4 days.



a *b* *c*

TEXT-FIG. 12, *a* to *c*. Temperature curves of unvaccinated controls. (*a*) Rabbit 17. (*b*) Rabbit 18. (*c*) Rabbit 19.

The pleural fluid contained a few leucocytes on the 2nd day, but was clear on the 4th day. Death intervened 4½ days after intratracheal injection. Only a few drops of fluid were present in the pleural cavity on autopsy. The pericardium was free from exudate. The pleura overlying the right lung contained a few patches of grayish white exudate. The consolidation was confined to the ventral lobe and the cephalic four-fifths of the caudal lobe. On section the involved portions varied from reddish gray to gray in color. Sections examined microscopically revealed the oldest lesions in the center of the ventral lobe. Here the consolidation was essentially gray.

Rabbits 17, 18, and 19.—(Text-fig. 12, *a*, *b*, and *c*.) All served as controls. Each received an intratracheal injection of 1/100 cc. of a 24 hour bouillon culture. All developed septicemia and died without particular lung involvements.

It was hoped by further experimentation along similar lines that the experimental disease could be still further varied by increasing the amount of vaccine or the time interval between vaccination and intratracheal infection. On the whole the results obtained were similar to those recorded. In a number of instances more diffuse pneumonias were produced, but even slight departures from the procedures previously described may so enhance the resistance that the animal fails to become infected. As additional evidence a brief record of another experiment is given, which illustrates about the maximum lung involvement obtained in any of the animals.

Rabbits 20 and 21.—(Text-figs. 5 and 6.) Each received a subcutaneous injection of vaccine. Rabbit 20 was injected intratracheally with living culture 7 days later. Rabbit 21 received the intratracheal injection 10 days later. Both reacted severely and succumbed 73 and 74 hours after infection. Necropsy of Rabbit 20 showed consolidation of the ventral and azygos lobes and the cephalic one-fourth of the caudal lobe of the right lung. In addition two round, grayish white consolidated areas were observed in the center of the caudal lobes. The consolidation was more pronounced in the case of Rabbit 21. The cephalic and ventral lobes of both lungs and the azygos were completely hepatized. The cephalic half of the right caudal lobe was also consolidated.

All rabbits were bled before vaccination and just before intratracheal injection. The results of the agglutination tests were not definite in serum dilutions as low as 1:1. In certain instances the serum was tested for opsonin. The serum of rabbits vaccinated 2, 3, or 4 days before infection failed to show any appreciable increase in opsonin. Tests of the serum from rabbits vaccinated 7 and 10 days prior to bleeding gave more definite results. In these observations about 60 per cent of the leucocytes contained the organisms. The tests were made by adding equal parts of a moderately heavy salt solution suspension of the organism to an equal part of the rabbit serum. After 2 hours' incubation a suspension of leucocytes obtained from a guinea pig was added. In addition a little fresh guinea pig serum was employed as complement. The whole was mixed and incubated for 2 hours. Films were made from the sediment.

DISCUSSION.

The results obtained by Wadsworth for the pneumococcus agree with those obtained in the experiments cited. Wadsworth injected rabbits with the pneumococcus by means of a needle introduced through the tracheal wall. He states that the inoculations led to variable results, either the animals were unaffected or they developed a rapidly fatal septicemia. In later experiments he first immunized rabbits with various products of the pneumococcus and in certain instances employed living cultures. After definite periods the animals were injected through the walls of the trachea with a highly virulent culture. Only a few manifested acute symptoms, but when killed 3 and 4 days after the infection pneumonia was found on autopsy.

In the writer's series of observations the lesions have been more pronounced. The rabbit apparently possesses little natural resistance for certain of the Pasteurella, so that at will one may so stimulate the resistance artificially that a variety of processes may be encountered. The normal rabbit reacts characteristically. The organism is extremely invasive and makes its way into the circulation from the lungs. The process is radically changed by the acceleration of the animal's resistance. The invasiveness of the organism is checked for a time and the process remains local. The principal lesion is that of fibrinous pneumonia with severe involvement of the bronchial epithelium. Rabbits whose resistance has been increased to a greater degree live longer. In these cases the alveolar exudate is fibrinopurulent or purulent. The epithelium of the air passages is severely involved. Perhaps during localization the protective substances are consumed and the organisms gain access to the circulation. From the results obtained one is inclined to believe that in animals possessing only a slight resistance the localizations occur close to the point of entry and are manifested as consolidations of the more dependent lobes. In animals of greater resistance the organisms evidently do not cause the pronounced reaction at the point of entry. The bacilli, however, to judge by the microscopic processes, invade the lymph channels and make their way to the pleura, but even in such conditions the process is essentially local. The blood remains sterile although the organisms exist in the pleural exudate in large numbers. Attempts to produce a long standing empyema or a more acute

pneumonia with resolution have failed. The relatively great invasiveness of the organism, its power to produce large necrotic foci, and the rather poor general resistance of the rabbit may offer a partial explanation.

To what degree resistance under natural conditions has so modified the type of disease resulting from respiratory infection can only be surmised. The experimental evidence is suggestive that organisms capable of producing a rapidly fatal septicemia may be made to localize within the chest cavity for definite periods and leads one to believe that organisms now associated with pneumonia are encountering a more resistant host which localizes their greatest activities at or near the site of infection. Certain of Blake and Cecil's[9] findings in their study of experimental pneumonia in monkeys suggest that even in such a highly susceptible species as the monkey considerable variations in natural resistance occur. Among a larger series of monkeys which were inoculated through the walls of the trachea with a Type I pneumococcus, two died of septicemia without particular lung involvement. A few others were entirely resistant, but a great proportion developed pneumonia. It is well recognized that certain of the Pasteurella do under natural conditions give rise to septicemia and are frequently associated with pneumonia. In the instance of bubonic plague, two well recognized types of disease exist, the septicemic form in which the organisms are introduced beneath the skin and the pneumonic type. It is probable that in the latter type the respiratory tract is the portal of entry and the organisms are localized for a time within the lung.

The distribution of the experimental pneumonia is similar to that encountered in natural infections in animals with organisms of this group. The infection apparently involves first the more dependent portions of the lungs, such as the ventral and cephalic lobes, and spreads from them. The pathology is essentially the same in both spontaneous and experimental disease. The consolidations vary from red to gray. Pleuritis and edema are also common to both.

One or two other points should be emphasized. The protocols in the first experiment indicate that the animals by vaccination with killed cultures may be made completely resistant to a subsequent inoculation into the trachea. It is to be inferred that the infecting

[9] Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 403.

doses in the experiments contain many more organisms than are introduced into the lower respiratory tract under natural conditions. That this immunity does not necessarily imply complete resistance when the organisms are artificially introduced beneath the integument, as in the case of subcutaneous injections, is also recorded in the protocols. It seems reasonable to presume that under natural conditions animals may be made greatly resistant to natural respiratory infections by vaccination. Protective substances are evidently formed with considerable rapidity, since the rabbit vaccinated only 2 days before infection proved more resistant than the unvaccinated control. The extreme delicacy of the balance between infection and resistance in experimental respiratory disease is illustrated in the instance of Rabbit 11 vaccinated 7 days before intratracheal injection. That the animal possessed sufficient resistance to prevent active infection is shown by subsequent injection of a larger dose of culture.

SUMMARY.

An organism of the Pasteurella group (*Pasteurella avicida*) has been shown to possess considerable pathogenicity for rabbits when injected into the trachea. 1 cc. of bouillon containing 1/100 cc. of a 24 hour bouillon culture introduced by means of a curved metal tube passed through the larynx gives rise to a rapidly fatal septicemia in which the lungs are not particularly involved. Rabbits may be made completely resistant to this intratracheal infection by means of intratracheal, intraperitoneal, or subcutaneous injections of killed cultures. Vaccination and subsequent injection of living culture beneath the skin resulted in extensive abscess formation about the site of inoculation. Vaccines sprayed on the mucous membranes of the nose and throat failed to raise appreciably the resistance against subsequent intratracheal injection.

Rabbits whose resistance has been stimulated within narrow limits by a small dose of vaccine beneath the skin become more resistant to the experimental respiratory infection. The character of the disease is changed from a rapidly fatal septicemia without lung involvement to a more prolonged pneumonia. The blood remains sterile for varying periods. Consolidations of the more dependent lobes occur with considerable regularity. In two instances a characteristic empyema developed in the course of the experiments.

STUDIES ON OXIDATION AND REDUCTION BY PNEUMOCOCCUS.

IV. Oxidation of Hemotoxin in Sterile Extracts of Pneumococcus.

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INTRODUCTION.

In 1912 Cole (1) described the presence of a hemotoxin in pneumococcus extracts and later (1914) (2) defined many of the biological properties of this substance. Pneumococcus hemotoxin is intracellular in nature, exists preformed in the bacterial bodies, and is liberated only after rupture and dissolution of the cells. The earlier study was carried out on bile or cholate solutions of pneumococci. The hemotoxin in these bacterial solutions which produced acute death in guinea pigs was found to cause rapid hemolysis of red blood corpuscles. In the later experiments, Cole confirmed these observations and brought convincing evidence that this hemolysin is an intracellular constituent of pneumococcus, and is unassociated with the action of the bile salts previously used, since he found it present in bacterial autolysates and in saline extracts prepared from washed, frozen, and ground pneumococci. The substance in pneumococcus extracts upon which this hemolytic property depends was found to be labile; its activity was destroyed in extracts heated to 55°C.; greatly decreased or completely lost by passage through a Berkefeld filter; destroyed by digestion with trypsin, and markedly inhibited by normal rabbit serum. Cole also observed that the hemolytic power of autolytic extracts was markedly diminished after 18 hours and completely absent after 48 hours exposure at 37°C.

In a preceding paper of this series on oxidation and reduction by pneumococcus, it has been demonstrated that sterile extracts of pneumococci are markedly reactive with molecular oxygen and that

peroxide is formed as a result of this union. These studies have afforded an opportunity to inquire whether these oxidative reactions have any effect upon the presence and potency of the hemotoxin contained in pneumococcus extracts.

EXPERIMENTAL.

Method.

Sterile Extracts of Pneumococcus.—The pneumococcus extracts were prepared by the methods described in the preceding papers (3, 4). They were of two types: (1) unwashed pneumococci extracted in broth; (2) washed pneumococci extracted in phosphate solution. In most instances these extracts were filtered through Berkefeld candles before use. All extracts employed in these tests were proved sterile by cultural and animal methods.

Hemolysis.—The hemolytic reaction of the extracts was tested as follows: The red blood corpuscles were removed by centrifugation from sterile, freshly drawn, defibrinated rabbit blood, washed three times, and finally suspended in salt solution. To 2.5 cc. of the 1 per cent suspension of red cells 0.1 cc. of the hemolytic extract was added. The mixture of red blood corpuscles and extract was incubated 3 hours at 37°C. The degree of hemolysis was recorded after incubation and final readings were made at the end of 12 hours in ice box.

Experiment 1. To Determine the Hemolytic Power of Pneumococcus Extract.—The extract used in the following experiment was prepared from Pneumococcus Type II. The extraction was carried out in the supernatant broth in concentration such that 1 cc. of the extract represented the extractable material from the bacterial cells in 35 cc. of the original broth culture. The bacteria were grown anaerobically. During the process of extraction the preparation was subjected to the minimal exposure to air, to prevent oxidation reactions, which will later be shown to affect materially the potency of the hemotoxin. This extract was passed through a Berkefeld filter to remove all formed elements, filtration being carried out in a nitrogen atmosphere. The final extract was preserved in the reduced state under heavy vaseline seal. The hemolytic titer of this extract was determined by finding the minimum amount of extract which would cause lysis of a constant suspension of red blood corpuscles.

From Table I it is apparent that as little as 0.001 cc. of the filtered, reduced extract caused marked but incomplete hemolysis of the red cells of rabbit blood and that 0.01 cc. of extract brought about complete cell solution. The minimal lytic amount of extract effecting complete hemolysis of a standard suspension of blood corpuscles represents, in this particular instance, the hemolytic substance extractable from the bacterial cells in 0.35 cc. of original culture. Con-

siderable loss of the hemolytic property of these extracts occurs during the procedures incident to their preparation. As pointed out by Cole the hemolytic effect of an extract is markedly diminished by Berkefeld filtration.

Experiment 2. To Determine the Effect of Heat on the Hemolytic Action of Pneumococcus Extract.—An unfiltered portion of the extract used in Experiment 1 was employed in this test. Equal quantities of the unfiltered, reduced extract were placed in a series of small narrow tubes and immediately sealed with vaseline. The various fractions of the extract were heated by immersing the sealed tubes for 10 minutes in water baths at 45°, 50°, 55°, and 65°C., respectively. Titrations of the hemolytic power of the heated and unheated extract were made by the method already described.

TABLE I.

Hemolytic Activity of Filtered, Reduced Broth Extract of Pneumococcus.

Amount of extract.	Hemolysis.
cc.	
0.10	++++*
0.05	++++
0.01	++++
0.005	+++
0.001	++

* ++++ = complete hemolysis.
+++ = almost complete hemolysis.
++ = incomplete hemolysis.
+ = slight "
− = no "

The effect of heating a sterile broth extract of pneumocccous for 10 minutes at temperatures ranging from 45°–65°C. is shown in Table II. Exposure for 10 minutes to 55°C. completely destroyed the hemolytic activity of the extract. This heat sensitiveness of the substance upon which hemolysis depends, is identical with that described by Cole for pneumococcus hemotoxin. The destruction of the reactive substance at 55°C. is evidence of its thermolability. A rapid increase in the rate of destruction occurs between 50° and 55°C. In a preceding paper it is pointed out that a similar acceleration in the rate of destruction of the peroxide-forming activity of pneumococcus extracts occurs between 60° and 65°C.

In previous communications it was shown that sterile broth extracts of pneumococci which have been preserved in the reduced state rapidly form peroxide when exposed to molecular oxygen. Moreover, with the accumulation of this compound the oxidized extract suffers a decrease and final loss of both its peroxide-forming and methylene blue-reducing activity. It seemed of interest, therefore, to study the effect of oxidation upon the hemolyzing action of a broth extract. Accordingly the following experiment was carried out.

Experiment 3. To Determine the Influence of Oxidation upon the Hemolytic Activity of a Broth Extract of Pneumococcus.—A comparison of the hemolytic activity of the same extract in the oxidized and reduced state was made in the following manner.

TABLE II.

The Effect of Heat upon the Hemolytic Activity of a Sterile Broth Extract of Pneumococcus.

Amount of extract.	Hemolysis by				
	Unheated extract.	Extract heated 10 min. at			
		45°C.	50°C.	55°C.	65°C.
cc.					
0.2	++++	++++	++++	−	−
0.1	++++	++++	++++	−	−
0.05	++++	++++	++++	−	−
0.01	++++	++++	++++	−	−
0.005	++++	+++	++	−	−

A sterile broth extract, which when kept in the reduced state exhibited marked hemolytic properties, was divided into three portions. One part was placed in a shallow layer in an Erlenmeyer flask and exposed to air at 37°C. in the dark. A second fraction was similarly treated except that it was exposed to air at 2°C. The third portion was transferred to a narrow tube, immediately sealed with vaseline, and held at 37°C. At the end of 1, 2, and 3 days, given amounts were removed from each of the three portions, and the hemolytic power of the oxidized and reduced fractions compared (see Table III).

The hemolytic power of a broth extract of pneumococcus differs, depending upon whether the extract is in the reduced or oxidized form. In Table III it is seen that if an extract is exposed to air it suffers loss of its hemolytic potency. On the other hand this property

may be conserved for considerable periods of time if the extract is kept in the reduced state unexposed to molecular oxygen. The reduced extract, after having been stored for 3 days at 37°C., caused prompt and complete lysis of the red blood cells in amounts of 0.01 cc. On the other hand, this amount of the same extract, after having been oxidized by exposure to air at 37°C. for 24 hours, failed to show any evidence of hemolysis when subsequently added to blood corpuscles. Active broth extracts of pneumococcus have been shown to unite with or accept oxygen when exposed to air. During and probably as a result of this oxidative reaction, peroxide is formed; on continued exposure to air, however, the extract loses its peroxide-

TABLE III.

Comparison of Hemolytic Activity of Reduced and Oxidized Sterile Broth Extract of Pneumococcus.

Amount of extract.	Hemolysis by								
	Reduced extract at 37°C. for days			Extract oxidized by exposure to air					
				At 37°C. for days			At 2°C. for days		
	1	2	3	1	2	3	1	2	3
cc.									
0.01	++++	++++	++++	−	−	−	−	−	−
0.005	+++	+++	+++	−	−	−	−	−	−
0.001	++	++	++	−	−	−	−	−	−

forming activity. This loss is the direct result and natural sequence of the oxidation of the extract. However, since the mechanism of hemolysis itself is not so easily referable to oxidation-reduction processes, it seems reasonable to assume that the hemolytic substance is indirectly destroyed by active products which are formed during the oxidation of other constituents of the extract. Since peroxide is known to accumulate during oxidation of a broth extract of pneumococcus, the effect of adding hydrogen peroxide directly to a sterile extract unexposed to air was determined in the following experiment.

Experiment 4. To Determine the Influence of Hydrogen Peroxide on the Hemolytic Activity of Sterile Broth Extracts of Pneumococcus.— The destruction of diphtheria and tetanus toxins by hydrogen peroxide

has been reported by Löwenstein (5) and by Sieber (6). Tests of the action of hydrogen peroxide upon pneumococcus hemotoxin seemed pertinent to a study of the nature of the reaction by which the hemolytic substance is destroyed, not only because peroxide is formed during oxidation of the extract, but because hydrogen peroxide is itself an active, chemical oxidizing agent.

M/3 and M/20 dilutions of titrated "Dioxygen" were prepared in sterile phosphate solution, pH 7.5. To 0.6 cc. of reduced broth extract of pneumococcus an equal amount of diluted hydrogen peroxide was added. The tubes containing the peroxide and extract mixture were immediately sealed with vaseline. The extract was exposed to the action of the hydrogen peroxide in final concentrations of 0.08 and 0.5 per cent for 4 hours at room temperature. At the end of this period the hemolytic titer of the peroxide-treated extract was compared with that of the untreated extract. As control, hydrogen peroxide in similar concentration was added to plain broth, and treated in the same manner as the extract to determine any possible action of the peroxide upon the red blood cells used in hemolysis test. These tests proved entirely negative and, therefore, are not included in the protocol.

The data presented in Table IV indicate clearly that reagent hydrogen peroxide destroys the hemolytic property of sterile broth extracts of pneumococcus. The untreated extract in amounts of 0.005 cc. caused complete hemolysis. Exposure of this same extract to 0.08 per cent of hydrogen peroxide for 4 hours so reduced the hemolytic activity that it required 0.1 cc. of the treated extract to effect an equal degree of hemolysis. In concentration of 0.5 per cent hydrogen peroxide destroyed completely the hemolyzing action of the extract. This is suggestive though not conclusive evidence that the destruction of the hemolytic substance, which occurs on exposing extracts of this type to the air, is related to the action of the peroxide which is formed during the oxidation of certain reactive constituents of the extract. Although the concentration of hydrogen peroxide added in this experiment is greatly in excess of the peroxide actually formed during oxidation of pneumococcus extract, it is well to remember that a great variety of higher organic peroxides may be formed as intermediate products in autoxidation, and that these substances are known to possess powerful oxidizing properties.

In the studies on oxidation and reduction by pneumococcus, the bacterial extracts employed were found to exhibit marked differences

in activity depending on whether or not they were prepared from washed or unwashed cells. If by the process of repeated freezing and thawing unwashed pneumococci are extracted in broth, the resulting extract possesses the properties of actively reducing methylene blue and of combining directly with molecular oxygen to form peroxide. It must be borne in mind that it is in extracts of this type that destruction of pneumococcus hemotoxin has been found to occur. Under these circumstances the actual destruction of the hemolytic substance may itself be the result of an oxidation process induced by oxidizing agents (peroxides) which are formed by the union of molecular oxygen with the autoxizable substances in the cell extracts.

TABLE IV.

Destruction of the Hemolytic Activity of Sterile Broth Extracts of Pneumococcus by Hydrogen Peroxide.

Amount of extract.	Hemolysis by		
	Untreated reduced extract.	Same extract after 4 hrs. exposure to H_2O_2.	
		0.08 per cent.	0.5 per cent.
cc.			
0.1	++++	++++	−
0.05	++++	+++	−
0.01	++++	+	−
0.005	++++	−	−

On the other hand, preparations obtained by extracting washed pneumococci in phosphate solution have been found incapable of inducing oxidation or reduction unless activated by the addition of cell washings, yeast extract, or broth. Since these saline extracts of washed pneumococci are by themselves not reactive with molecular oxygen, slight or no oxidative reactions occur in them on exposure to air. Pneumococcus hemotoxin in extracts of this type might be expected, therefore, to be more stable in the presence of molecular oxygen. To ascertain whether this is actually the case the following experiment was carried out.

Experiment 5. Comparison of the Effect of Exposure to Air on the Hemolytic Activity of Broth Extracts of Unwashed Pneumococci, and Saline Extracts of Washed Cells.—Unwashed cell extract: prepared as previously described (3) by extraction

of unwashed pneumococci in broth. This extract reduced methylene blue and formed peroxide on exposure to air.

Washed cell extract: prepared as previously described (3) by extraction of washed pneumococci in phosphate solution. This extract did not reduce methylene blue or form peroxide until activated by the addition of yeast extract or broth.

Exposure to air: each preparation was exposed to the air in shallow layer in an Erlenmeyer flask at 37°C.

Titration of hemolysis: at stated intervals portions of each extract were removed and the hemolytic activity determined by the method described. The results are given in Table V and presented graphically in Text-fig. 1. In the latter the logarithms of the number of "hemolytic units" in 1 cc. of extract are plotted against the hours of exposure to air.

TABLE V.

The Effect of Exposure to Air upon the Hemolytic Activity of Extracts of Unwashed and Washed Pneumococci.

1. Broth extract of unwashed pneumococci.

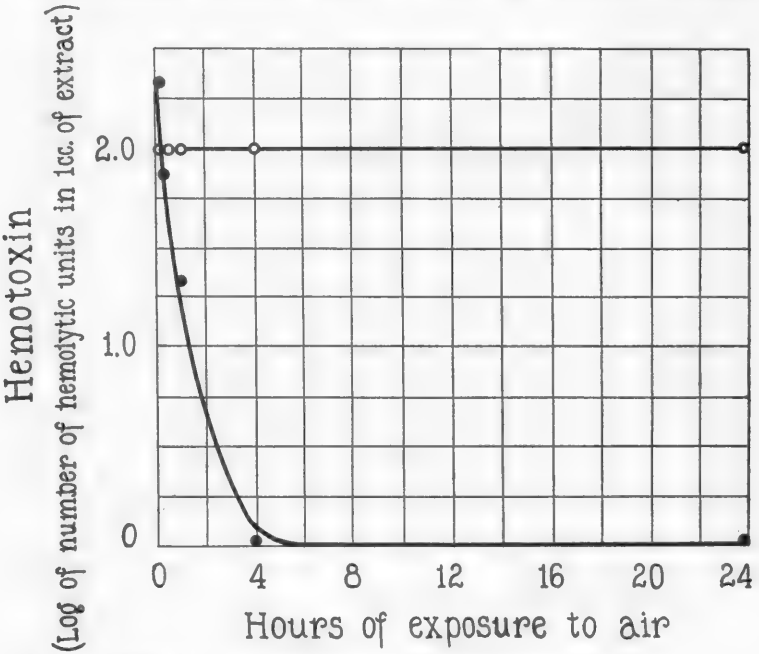
Amount of extract.	Hemolysis by					
	Extract not exposed to molecular oxygen.	Extract after exposure to molecular oxygen at 37°C. for				
		30 min.	1 hr.	4 hrs.	24 hrs.	96 hrs.
cc.						
0.05	++++	++++	+++	±	−	−
0.01	++++	+++	+	−	−	−
0.005	++++	±	+	−	−	−

2. Saline extracts of washed pneumococci.

cc.						
0.05	++++	++++	++++	++++	++++	+++
0.01	+++	+++	+++	+++	+++	+
0.005	+	+	+	+	±	−

Pneumotoxin is an intracellular substance which is liberated from the bacterial bodies whenever the cell undergoes dissolution. This hemolytic agent is present in pneumococcus extracts prepared both from washed and unwashed cells. The occurrence of an easily detectable hemolysin in these two kinds of extract, one type of which is reactive with molecular oxygen and the other wholly unaffected by exposure to air, afforded an opportunity to determine the effect of oxidation upon the stability of this hemotoxin. The results of an experiment of this nature are recorded in Table V. It is evident

that in the absence of air the hemotoxin is remarkably stable in both types of extract. In the presence of molecular oxygen, however, the stability of the hemolytic substance is different in each type of extract. In broth extracts of unwashed cells, which contain oxygen-reactive substances, and in which the oxidation processes



● = unwashed cell extract.
○ = washed cell extract.

TEXT-FIG. 1. Effect of exposure to air upon the hemolytic activity of extracts of unwashed and washed pneumococci.

result in the formation of peroxide, the hemotoxin rapidly disappears when the extract is exposed to air. On the other hand, in saline extracts of washed cells which by themselves are not reactive with molecular oxygen, this hemolytic substance remains practically unchanged in activity even after prolonged exposure to air.

This latter fact is evidence that the hemolytic substance as such is not affected by oxygen, even in the presence of the enzymes in washed cell extracts. It is evident that pneumococcus hemotoxin, a substance not reactive with molecular oxygen, is destroyed in extracts only in the presence of other constituents which are capable of undergoing autoxidation.

SUMMARY.

In the present work on oxidation and reduction by sterile extracts of pneumococcus, the preparations employed contain among other constituents, a hemolytic substance the properties of which have been described by Cole (1, 2) in his studies on pneumococcus hemotoxin. Pneumococcus extracts prepared by the methods described are actively hemolytic, 0.005 cc. of extract causing complete lysis of 2.5 cc. of a 1 per cent suspension of red cells from rabbit blood. This hemolytic property of pneumococcus extracts is destroyed by 10 minutes exposure to 55°C. When pneumotoxin-containing extracts are protected from the action of molecular oxygen, their hemolytic activity remains unimpaired for considerable periods of time. In the presence of air, on the other hand, the stability of the hemolytic substance depends upon whether the particular type of extract contains a "complete" or "incomplete" oxidation-reduction system. Sterile broth extracts of unwashed pneumococci are reactive with molecular oxygen, and as a result of this union peroxide is formed whenever these extracts are exposed to air. The hemolytic activity of "complete" extracts of this type is rapidly decreased and finally destroyed in the presence of molecular oxygen. On the other hand, the "incomplete" type of extract prepared by saline extraction of washed pneumococci may be exposed to air with little or no loss of hemolytic power. This "incomplete" washed cell extract, unless reactivated, does not undergo autoxidation in the presence of air; under these circumstances peroxide is not formed and the hemolytic activity of this type of extract is not impaired by exposure to air. The stability of the hemolytic agent in the "incomplete" type of extract is evidence that this substance is itself not reactive with or affected by molecular oxygen, even in the presence of the cell enzymes. The destruction of the same hemolytic substance in extracts capable

of undergoing autoxidation may be ascribed to the action of some peroxide formed by the union of molecular oxygen with easily oxidized or autoxidizable substances of the extract. It is now known that a peroxide, having the reactions of hydrogen peroxide, accumulates in sterile pneumococcus extracts during oxidation. It has been shown in the present study that the addition of preformed hydrogen peroxide destroys the hemolytic activity of pneumococcus extracts, although higher concentrations were required than were detected in oxidized extracts themselves. These facts and the known action of superoxides in analogous types of reaction make it seem not unlikely that the active agent in the destruction of pneumotoxin in oxidized cell extracts may be a peroxide; either hydrogen peroxide or some higher organic peroxide formed during autoxidation of the extract.

CONCLUSIONS.

When pneumococcus extracts are exposed to air, the hemotoxin is destroyed only in those extracts capable of undergoing autoxidation. The active agent responsible for this destruction is probably a peroxide formed by the union of molecular oxygen with some other, easily oxidizable constituents of the extract.

BIBLIOGRAPHY.

1. Cole, R., *J. Exp. Med.*, 1912, xvi, 644.
2. Cole, R., *J. Exp. Med.*, 1914, xx, 346.
3. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 357.
4. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 543.
5. Löwenstein, E., *Wien. klin. Woch.*, 1903, xvi, 1393.
6. Sieber, N., *Z. physiol. Chem.*, 1901, xxxii, 573.

STUDIES ON OXIDATION AND REDUCTION BY PNEUMOCOCCUS.

V. The Destruction of Oxyhemoglobin by Sterile Extracts of Pneumococcus.

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In the first of this series of papers suspensions of living pneumococci, which during growth had been protected from the action of air by anaerobic cultivation, were shown to possess the property of uniting with molecular oxygen to form peroxide when exposed to air under conditions unfavorable for cell multiplication (1). The three succeeding papers have dealt with the formation of peroxide (2), the reduction of methylene blue (3), and the oxidation of hemotoxin (4) by sterile extracts of pneumococci under conditions excluding the participation of living or intact cells in these oxidative reactions.

The present paper is concerned with another activity of reduced extracts of pneumococci: the destruction of oxyhemoglobin and the formation of methemoglobin. The principal object of this study is the attempt to obtain more knowledge concerning the mechanism of the destruction of oxyhemoglobin by pneumococcus, and to correlate, if possible, the nature of this phenomenon with the other known oxidation-reduction activities of pneumococcus extracts.

EXPERIMENTAL.

Methods.

Bacteriological.—The sterile extracts of pneumococci were prepared by the methods described in a preceding paper (2). Sterile broth extracts of unwashed pneumococci were used in the following experiments unless otherwise stated. All of the extracts used were proved sterile by cultural and animal tests. These extracts were prepared from anaerobically grown pneumococci and during their preparation were protected from the action of air by being kept under a heavy vaseline seal. Extracts which have been stored in sealed tubes protected from air are referred to in the text as reduced extracts.

Chemical.—The crystalline hemoglobin was prepared from horse blood by Heidelberger's method (5). The hemoglobin crystals were dissolved in 0.1 M phosphate solution (pH 7.5) and the solutions passed through Berkefeld filters. The oxyhemoglobin solutions from laked washed red cells were prepared without crystallization from sterile blood. The spectroscopic examinations were made with a prism spectroscope. The oxyhemoglobin analyses were made by the oxygen capacity method of Van Slyke (6), with the apparatus and technique described by Van Slyke and Neill (7). In determining the "total hemoglobin" the colorimetric method of Stadie (8) was used.

Destruction of Oxyhemoglobin by Sterile Extracts of Pneumococci.

Although a few investigators have demonstrated the production of methemoglobin by sterile extracts and culture filtrates of pneumococci, others have been unable to obtain this reaction in the absence of the living organism. The literature on this subject has been reviewed by Butterfield and Peabody (9), Cole (10), Stadie (11), Schnabel (12), and others.

In the present study, sterile reduced extracts of unwashed pneumococci prepared as described, have been found to induce marked and rapid pigment changes when added to blood or to hemoglobin solutions. Methemoglobin production is evident in 30 minutes in tests in which 0.05 cc. of the extract is used. The greenish, discolored areas formed by pneumococci growing on blood agar plates can also be produced in the complete absence of living cells by placing a small portion of the sterile extract in a depression cut in a fresh blood agar plate.

Quantitative measurements show that the amount of oxyhemoglobin destroyed by these sterile extracts is quite significant. The addition of 0.5 cc. of the extract which has not previously been exposed to air, to 5 cc. of rabbit or human blood, causes a destruction of between 200 and 300 mg. of oxyhemoglobin within a few hours at 37°C.

Destruction of Oxyhemoglobin to Products beyond Methemoglobin.

The intensity of the reaction and the marked pigment changes induced by increasing amounts of active extract indicated that in many instances the destructive process may yield degradation products still lower than methemoglobin. To obtain quantitative evidence of this action, measurements were made of the "total hemo-

globin" (oxyhemoglobin, hemoglobin, and methemoglobin) in solutions to which large and small amounts of active extract had been added.

The data obtained in a number of experiments furnished quantitative evidence that sterile extracts of pneumococci, if present in high concentration, not only convert oxyhemoglobin to methemoglobin, but destroy these blood pigments to still lower degradation products. In low concentration the action of these same extracts is limited to the conversion of oxyhemoglobin to methemoglobin.

Oxyhemoglobin Destruction by Hydrogen Peroxide in the Presence and Absence of Catalase.

Hydrogen peroxide is a chemical agent which is known to effect many oxidations in much the same manner as do living cells. As the destruction of oxyhemoglobin by sterile pneumococcus extract is in all probability an oxidation reaction, it seemed of interest to compare the effect on hemoglobin of the reaction brought about by this chemical agent with that induced by the sterile bacterial preparation. This comparison acquires especial interest from the fact that a peroxide, having the reactions of hydrogen peroxide, is known to accumulate in media during the aerobic growth of pneumococcus. However, the following experiments are introduced here, not to emphasize the importance of hydrogen peroxide as such, but rather to compare the action of an active extract with that of a known oxidizing agent in the presence and absence of blood catalase.

Influence of Catalase in Protecting Oxyhemoglobin from the Action of Hydrogen Peroxide.—The crystalline hemoglobin used in these experiments was almost entirely devoid of catalase. On the other hand, the solutions of hemoglobin prepared directly from laked corpuscles were rich in blood catalase. From these two sources, therefore, it was possible to prepare solutions of equal hemoglobin concentration, which differed widely in their content of blood catalase. In previous experiments the presence or absence of catalase was found to have little or no effect on the oxyhemoglobin-destroying power of the reduced extract of pneumococcus, since practically identical results were obtained with solutions of oxyhemoglobin from laked cells containing abundant catalase and with solutions of crystalline hemoglobin containing only traces of catalase. Quite different results, however, might be expected if preformed hydrogen peroxide ("Dioxogen") were added directly to these two types of hemoglobin, since catalase is known to destroy hydrogen peroxide.

Experiments were made to contrast the hemoglobin-sparing action of catalase in the presence of hydrogen peroxide with the lack of this protection in the presence of reduced extract of pneumococcus; and

to compare the type of change in oxyhemoglobin brought about by the action of hydrogen peroxide with that induced by pneumococcus extract. Because of lack of space the detailed protocols of these experiments are omitted.

As might be expected, the presence of catalase greatly inhibits the action of hydrogen peroxide upon hemoglobin. It was found that hemoglobin, prepared from the crystalline product containing only traces of catalase, was entirely destroyed to colorless products by amounts of hydrogen peroxide which failed to produce any detectable change in solutions of the same concentration of hemoglobin having abundant catalase. The influence of catalase in protecting oxyhemoglobin from the action of hydrogen peroxide is in striking contrast to the almost complete indifference to catalase of the oxyhemoglobin-destroying power of reduced extracts of pneumococcus. However, in spite of this marked protective action of catalase, large amounts of hydrogen peroxide can destroy small amounts of hemoglobin with the production of methemoglobin.

Comparing the type of change in blood pigment effected by this chemical agent with that induced by bacterial extracts, reactions of a quite similar nature were found to occur in both instances. Increasingly large amounts of pneumococcus extracts caused an increasing and progressive degradation of hemoglobin to products beyond methemoglobin. Similar relations have been found to hold true for hydrogen peroxide. Moreover, in the presence of an excess of preformed hydrogen peroxide, the successive phases through which the reaction proceeds simulate most closely those brought about by the action of the bacterial extract upon hemoglobin.

These relations may be interpreted as evidence that the active substance involved in hemoglobin destruction by pneumococcus extract is an oxidizing agent which induces the same general type of reaction as does hydrogen peroxide. However, in the presence of blood catalase, very large amounts of preformed hydrogen peroxide are required; while the hemoglobin-destroying agent in pneumococcus extract is apparently indifferent to the action of catalase.

Influence of Heat on the Hemoglobin-Destroying Activity of Sterile Extracts of Pneumococcus.

The peroxide-forming and methylene blue-reducing activity of a sterile broth extract of unwashed pneumococci is completely destroyed by heating the extract at 65°C. for 10 minutes (3). In a number of experiments the heat stability of the methemoglobin-forming function of pneumococcus extract has been tested and compared with the heat sensitiveness of the system responsible for the formation of peroxide in the same extract.

If an extract is heated at a constant temperature (55°C.) for gradually increasing periods of time, from 10 to 60 minutes, there results a gradual and progressive loss in what may be termed the total oxidizing and reducing power of the extract; less peroxide is formed, methylene blue is more slowly and less completely decolorized, and the rate and degree of hemoglobin destruction decrease. Moreover, not only is the activity of these three functions destroyed at the same rate at 55°C. but the destruction of each shows the same marked acceleration between 60° and 65°C. These relationships, together with the nature of the processes induced, indicate that these properties are functions of the same or closely related systems in the extract.

Activation of the Hemoglobin-Destroying Power of Sterile Extracts of Washed Pneumococci.

In the experiments thus far recorded in this paper, sterile broth extracts of unwashed pneumococci have been used exclusively. The results recorded are sufficient evidence of the marked hemoglobin-destroying activity of extracts of this type. It will be recalled that extracts of washed cells in phosphate solutions have been found to contain potential but incomplete oxidation-reduction systems, and that extracts of this type form peroxide (2) and reduce methylene blue (3) only if they are activated or completed by the addition of cell washings, meat infusion, or yeast extract. In view of the close relationships which seem to exist between these various functions, it might be expected that hemoglobin destruction by washed cell extracts would also be dependent upon the presence of these complementing substances.

Analysis of Table I reveals the interesting fact that an extract of washed pneumococci is unable by itself to destroy hemoglobin, and that this same extract when activated or completed by the addition of certain complementary substances rapidly converts the oxyhemoglobin to methemoglobin. It is evident that yeast extract and muscle infusion complete some otherwise deficient system in the washed cell extract and thus activate the function upon which oxyhemoglobin destruction depends. These relations are analogous to the completion in the same extracts of the peroxide-forming and methylene blue-reducing systems. In an earlier study on the for-

TABLE I.

Activation of the Oxyhemoglobin-Destroying Power of Sterile Extracts of Washed Pneumococci.

Saline extract of washed pneumococci.	PO_4 solution.	Meat infusion.	Yeast extract.	HbO_2 solution.	HbO_2 destruction and MetHb formation after 2 hrs. at 37°C.
cc.	*cc.*	*cc.*	*cc.*	*cc.*	
0.6	0.6	—	—	0.6	—
0.6	—	0.6	—	0.6	+
0.6	—	—	0.6	0.6	+
—	—	0.6	—	0.6	—
—	—	—	0.6	0.6	—

mation of methemoglobin by pneumococcus extracts Avery and Cullen[1] found that an acetone-ether extract of muscle infusion or serum contained substances which served to activate the methemoglobin-forming system of washed bacterial extracts. Although in the present study no attempt has been made to identify further the nature of these substances, it is possible that they may be related to the acetone-ether soluble substances which Meyerhof (13) has shown to be important in the autoxidation processes of tissue cells. However, it is not unlikely that meat infusion broth and yeast extract contain other types of autoxidizable substances.

[1]Unpublished observation.

Comparison of the Amount of Oxyhemoglobin Destroyed by Hydrogen Peroxide and by Reduced and Oxidized Extracts of Pneumococcus.

Throughout these studies the extracts used have been prepared from anaerobically grown cultures of pneumococci and care has been taken to exclude air during the processes of extraction. Sterile broth extracts of unwashed cells prepared under these precautions and preserved under seal are referred to as reduced extracts. Whenever a reduced extract of this type is exposed to air, oxidation products are formed which in turn react upon other constituents of the extract. Extracts in which these changes have been induced by exposure to air are spoken of as oxidized. In preceding papers (3, 4) marked differences have been shown to exist in the activity of the same extract in the reduced and oxidized form.

It seemed of interest to determine the effect of oxidation upon the hemoglobin-destroying activity of an extract. For purposes of comparison, the amount of oxyhemoglobin destroyed by an excess of preformed hydrogen peroxide is contrasted with the amount of blood destruction induced by the pneumococcus extract. This comparison is pertinent since peroxide is formed whenever an extract passes from the reduced to the oxidized form. Moreover, it furnishes quantitative proof of the fact that while blood catalase seriously impairs the action of hydrogen peroxide on hemoglobin, it exerts little or no effect on the hemoglobin-destroying activity of reduced extracts of pneumococcus.

Sterile broth extract of pneumococcus which had been preserved in the reduced form was divided; an aliquot portion of the reduced extract was exposed to the air at 20°C. for 7 hours. This oxidized extract contained demonstrable amounts of peroxide, which had formed during the process of oxidation. The hydrogen peroxide consisted of titrated dilutions of "Dioxogen" in concentration of 1.12 M and 0.28 M solutions. Sterile, freshly defibrinated rabbit blood in which the red cells were hemolyzed by freezing and thawing was added in 2 cc. amounts to six tubes; to each of the first two, 3 cc. of a known dilution of hydrogen peroxide were added; in the next two, 0.4 cc. of reduced and oxidized extract was placed respectively. The last two served as controls. In each instance the total volume was made up to 6 cc. by the addition of broth, phosphate solution, or water as required. All tubes were incubated at 37°C. for 6 hours, and then the oxyhemoglobin content of the series was determined. One of the control tubes containing diluted blood was held at 2°C. until the end of the experiment as a control of the

initial HbO_2. The loss of HbO_2 in the control during incubation at 37°C. was subtracted from the total loss of HbO_2 in all samples. The results are given in Table II.

In terms of the amount of hemoglobin destroyed, Table II presents two striking contrasts; first, the disparity of action between reduced pneumococcus extract and preformed hydrogen peroxide, and second, the extreme difference in the activity of the reduced and oxidized extract. The first contrast is strikingly brought out by assuming, for the purposes of comparison, that the hydrogen peroxide formed during oxidation of the reduced extract is actually concerned *per se* in the destruction of the blood pigment. If the

TABLE II.

Comparison of the Amount of Oxyhemoglobin Destroyed by Hydrogen Peroxide and by Reduced and Oxidized Extracts of Pneumococcus.

Sterile defibrinated rabbit blood.	Amount of hydrogen peroxide (Dioxogen).	Pneumococcus extract.		Amount of oxyhemoglobin destroyed in 6 cc.	
		Reduced.	Oxidized.		
cc.	*millimols*	*cc.*	*cc.*	*millimols*	*mg.*
2	3.36	—	—	0.015	25
2	0.84	—	—	0.008	14
2	—	0.4	—	0.072	121
2	—	—	0.4	0.003	5
2	—	—	—	—	—

production of one molecule of H_2O_2 requires the utilization of one molecule of O_2, then it would be necessary for 0.4 cc. of the reduced bacterial extract to utilize 75,300 c.mm. of oxygen to produce 3.36 millimols of hydrogen peroxide, an amount equal to that added in the experiment. However, the addition of this quantity of hydrogen peroxide resulted in the destruction of only 25 mg. of oxyhemoglobin. On the other hand, 0.4 cc. of the reduced extract destroyed 121 mg. of hemoglobin, approximately five times as much as that destroyed by 3.36 millimols of hydrogen peroxide. These figures do not exclude the possibility that hydrogen peroxide as such may function as the active agent in the destruction of hemoglobin by pneumococcus. However, if oxyhemoglobin destruction under these conditions is attributable to the action of the hydrogen peroxide formed by the

extract, then these figures furnish a striking example of the greater activity of this agent at the moment of its formation than when subsequently added to the system in a preformed state.

The second striking contrast revealed in Table II is the extraordinarily marked difference in activity exhibited by the same extract in the reduced and oxidized form. The reduced extract in amounts of 0.4 cc. destroyed 121 mg. of hemoglobin, while a like quantity of the oxidized extract destroyed only 5 mg., an amount so slight that it may be ignored, being not more than twice the experimental error involved in the analytical technique.

When a reduced extract of pneumococcus undergoes oxidation, its hemoglobin-destroying activity is rapidly lost. After 30 minutes aeration the extract contains demonstrable amounts of peroxide and shows a certain decrease in the rate of methemoglobin formation when added to blood. The loss in activity of the methemoglobin-forming system is progressive with increasing oxidation until at the end of 2 to 3 hours exposure to air, complete and final loss of the methemoglobin-forming power of the extract has occurred. During oxidation, peroxide accumulates in the extract but the peroxide formed in the oxidized extract if subsequently added to blood is unable by itself to effect any change in the pigment in the presence of blood catalase.

The destruction of hemoglobin, with the resulting formation of methemoglobin by sterile extracts of pneumococcus, is apparently a function of the oxidation-reduction systems contained in the extracts. An extract in which oxidation has occurred is subsequently incapable of converting oxyhemoglobin to methemoglobin.

On the other hand, an extract in which the active substances are protected from oxidation retains its potency for months, even if stored at 37°C. These facts indicate that methemoglobin formation is a process which accompanies and is dependent upon the initial oxidation of some constituent of the extract. Moreover, in the formation of methemoglobin, it is necessary that the oxyhemoglobin be present in the system during the oxidation of the extract; if an extract is completely oxidized before the addition of oxyhemoglobin the reaction does not occur in the presence of catalase.

Comparison of the Action of Reduced and Oxidized Extract of Pneumococcus on Oxyhemoglobin in the Presence and Absence of Blood Catalase.

In a preceding experiment it was shown that when hydrogen peroxide (reagent) is added to oxyhemoglobin, the pigment changes which result are determined by and dependent upon the balance between the concentration of the chemical agent and the amount and activity of the blood catalase present. In solutions of crystalline oxyhemoglobin containing little or no catalase, methemoglobin was rapidly formed by amounts of hydrogen peroxide which produced no change in catalase-containing solutions of red cells. In the latter instance the catalase in decomposing hydrogen peroxide served to protect the oxyhemoglobin, while in the former case the absence of catalase exposed the blood pigment to the direct action of hydrogen peroxide itself. On the other hand, reduced pneumococcus extract effects the transformation of oxyhemoglobin to methemoglobin in the presence as well as in the absence of catalase. This difference of behavior suggests that the mechanism of the latter reaction is more complex than the simple interaction between hydrogen peroxide and oxyhemoglobin. This assumption would be further strengthened if it could be shown that the peroxide which accumulates as a final product of the oxidation of pneumococcus extract were quite as readily destroyed by the catalase of the blood as is hydrogen peroxide when added directly as a chemical reagent. In order to test the validity of this point the following experiment was carried out.

Oxyhemoglobin containing only faint traces of catalase was obtained by preparing 0.4 mM solutions of the crystalline product in phosphate solution (pH 7.5). Oxyhemoglobin containing abundant catalase was obtained by the use of defibrinated blood undiluted or diluted tenfold in phosphate solution. A portion of the same extract as that used in the reduced state was exposed to air for 12 hours at 20°C. The oxidized extract at the end of this period and before oxyhemoglobin was added, gave a strong peroxide reaction. Portions of the oxidized and reduced extract were heated 10 minutes at 75°C. to inactivate the labile constituents of the extract. The oxidized extract after heating still gave a marked peroxide reaction. 0.1 cc. portions of heated and unheated extract, both reduced and oxidized, were added to 0.9 cc. of the catalase-free and catalase-rich solutions of oxyhemoglobin in the order presented in Table III. Spectroscopic examinations were made after 4 hours at 37°C.

This experiment shows that when reduced extract is added to defibrinated blood methemoglobin is formed in spite of the presence of catalase. Furthermore, the same pneumococcus extract if allowed to oxidize with the consequent production of hydrogen peroxide fails to induce methemoglobin formation when subsequently added to blood because of the capacity of the catalase present to destroy this

agent. That the hydrogen peroxide formed in the oxidized extract is active in the absence of catalase is easily shown by the facility with which it destroys crystalline hemoglobin. Moreover, to insure against the participation in this reaction of any active agent other than the hydrogen peroxide, the oxidized extract was completely inactivated by exposure to 75°C. for 10 minutes, without any effect upon its action on crystalline hemoglobin. Oxidized extract in which the final products of oxidation have accumulated behaves toward oxyhemoglobin, under these circumstances, precisely like a dilute

TABLE III.

Comparison of the Action of Reduced and Oxidized Extract of Pneumococcus upon Oxyhemoglobin in the Presence and Absence of Blood Catalase.

Pneumococcus extract.		Presence of peroxide in extract before adding HbO_2.	Methemoglobin formation.	
			Oxyhemoglobin from laked blood (abundant catalase).	Solutions of crystalline oxyhemoglobin (no catalase).
Reduced.	Unheated.	—	+	+
	Heated 10 min. at 75°C.	—	—	—
Oxidized.	Unheated.	+	—	+
	Heated 10 min. at 75°C.	+	—	+

solution of hydrogen peroxide; that is, it is unable to form methemoglobin in the presence of blood catalase and is no more heat-labile than is hydrogen peroxide itself.

Obviously, catalase can protect hemoglobin against the action of oxidized extract containing the final product, H_2O_2; but it offers no protection when the hemoglobin is present in the system *during* the oxidation of the extract. A probable explanation of the difference in activity under these two sets of circumstances is that during the process of oxidation of a pneumococcus extract certain intermediary products are formed which like hydrogen peroxide are strong oxidizing agents, but unlike this substance are not destroyed by catalase. If hemoglobin is present during the formation of these intermediary bodies, it is in turn oxidized by these substances. Presumably when the process of oxidation of pneumococcus extract has been completed,

these higher peroxides are converted into simple hydrogen peroxide the action of which, when subsequently added to oxyhemoglobin, is conditioned by the presence or absence of blood catalase.

Influence of Hydrogen Peroxide on the Oxyhemoglobin-Destroying Activity of Pneumococcus Extract.

When a reduced extract of pneumococcus is exposed to the air it suffers a rapid and progressive loss of its ability to form methemoglobin. During this oxidation hydrogen peroxide is formed and it was previously suggested that part of the loss of activity may be due to the destructive action of this oxidation product upon the extract. Evidence of the probable importance of the accumulated peroxide as a factor in the destruction of the methemoglobin-forming system in oxidized extracts has been furnished by a number of quantitative experiments. Treatment of active extracts with reagent hydrogen peroxide resulted in a complete loss of ability of the treated extract to destroy oxyhemoglobin.

Influence of the Oxygen Tension upon Methemoglobin Formation by Sterile Extracts of Pneumococci.

In an earlier investigation (1914) Cole (10) observed that the degree of oxygenation of the blood was an important factor in the formation of methemoglobin by living pneumococci. To obtain further evidence of the influence of the degree of oxygenation of hemoglobin upon methemoglobin formation it seemed desirable to repeat Cole's experiments with sterile extracts instead of living bacteria. In the following experiment, therefore, a sterile pneumococcus extract was added to and allowed to act upon hemoglobin in various degrees of oxygenation.

4.5 cc. of sterile defibrinated rabbit blood and 0.5 cc. of 0.1 M phosphate solution (pH 7.5) were placed in each of three tonometers of 300 cc. capacity. One of these samples of blood was then deoxygenated by evacuation and by preliminary saturation with hydrogen. Another was saturated with oxygen at 20 mm. tension and the third was saturated with oxygen at 710 mm. As a control 5 cc. of blood similarly diluted to which phosphate solution instead of extract was added served as control at atmospheric tension. After the preliminary saturations, sterile reduced extract in amounts of 0.8 cc. was added to each specimen of blood, precautions being taken to prevent any oxidation of the extract or any change in the composition of the gas during the manipulation.

The tonometers containing mixtures of sterile pneumococcus extract and of blood at different degrees of oxygenation, were then rotated in a water bath at

38°C. for 2 hours. The formation of methemoglobin in the different samples was then determined by measurements of the loss of oxygen capacity of the blood. The results are given in Table IV.

Table IV shows that when a mixture of reduced extract of pneumococcus and deoxygenated blood was exposed to an oxygen tension of less than 3 mm., little or no methemoglobin was formed. The actual figures in the table are not more than twice the experimental error and indicate that if the preliminary deoxygenation had been complete the hemoglobin would have suffered no change under

TABLE IV.

Influence of Oxygen Tension and Degree of Oxygenation of Hemoglobin upon the Formation of Methemoglobin by Sterile Extracts of Pneumococcus.

Oxygen tension in gas phase.	Degree of oxygenation of hemoglobin $\left(\text{Ratio } \frac{Hb}{Hb + HbO_2}\right)$.		Action of reduced extract of pneumococcus.	
	Hb	HbO_2	Hemoglobin content after 2 hrs. at 37°C. (oxygen capacity).	Hemoglobin destroyed (methemoglobin formed).
*mm.**	*per cent**	*per cent**	*mM*	*mg. in 5.8 cc.*
0	100	0	5.35	15
20	50	50	3.90	168
710	0	100	5.24	27
Control.			5.50	

* Approximate figures.

these conditions. When the reaction was carried out at high oxygen tensions, 710 mm., that is under conditions such that the concentration of deoxygenated hemoglobin was practically negligible, only a very small amount of methemoglobin was formed. At an oxygen tension of 20 mm., both oxyhemoglobin and deoxygenated hemoglobin were present in approximately equal amounts. When the extract acted upon blood under these conditions significant amounts of methemoglobin were formed as shown by the marked decrease in oxygen capacity. This oxygen tension was chosen at random and although serving the purpose of the experiment it may not represent the optimum for the reaction.

From the results of this experiment it may be concluded that the methemoglobin-forming reaction of pneumococcus is influenced by

oxygen tension in two ways. Thus, while the presence of oxygen is necessary for the formation of the oxidizing agent which oxidized hemoglobin to methemoglobin, the reaction does not proceed to advantage at high oxygen tensions which exclude the presence of hemoglobin in the deoxygenated form. As previously observed by Cole (10), the formation of methemoglobin by pneumococcus apparently involves a preliminary deoxygenation of hemoglobin. The present study suggests that this process of deoxygenation may have

TABLE V.

Oxygen Consumed in the Destruction of Oxyhemoglobin by Pneumococcus Extract.

	Oxygen capacity.			Oxygen content.		
	0	1 hr.	2 hrs.	0	1 hr.	2 hrs.
	mM	mM	mM	mM	mM	mM
Control.	6.5	6.5	6.5	6.5	6.4	6.3
Extract.	6.5	5.1	4.5	6.5	3.2	1.5
	Hemoglobin destroyed by extract.			Oxygen consumed by extract.		
	0	1.4	2.0	0	3.2	4.8

a twofold function; it affords a source of oxygen to the peroxide-forming system of the cell and in turn it leaves the hemoglobin in a form more readily oxidized to methemoglobin by the peroxide thus formed.

The Consumption of Oxygen during the Destruction of Oxyhemoglobin by Sterile Pneumococcus Extract.

From the results of the preceding experiments it was inferred that the oxygen united with some constituent of the extract to form an oxidizing agent which *oxidized* deoxygenated hemoglobin to methemoglobin. In view of these relations, experiments were conducted to correlate the amount of oxygen consumed with the amount of hemoglobin destroyed.

From the results given in Table V it appears that during the reaction between pneumococcus extract and oxygenated blood, the molecular amount of oxygen consumed is greater than that of hemo-

globin destroyed. From the previous experiment it was inferred that deoxygenated hemoglobin represents an intermediary product in the formation of methemoglobin. Although it is frequently impossible to demonstrate the presence of intermediary substances in biological oxidations, it is interesting to observe that deoxygenated hemoglobin accumulates in significant concentrations during this reaction, at least in systems in which oxyhemoglobin is the source of molecular oxygen.

DISCUSSION.

Sterile broth extracts of unwashed pneumococci actively destroy oxyhemoglobin with the resulting formation of methemoglobin. The rate and degree of destruction are largely dependent upon the concentration of the reagents used. Larger amounts of extract not only transform oxyhemoglobin to methemoglobin but may destroy the blood pigment to products beyond methemoglobin. This process seems to be a function of the same system or systems responsible for the formation of peroxide and the reduction of methylene blue by pneumococcus extract (3, 4). This fact is further evidenced by the heat sensitiveness of the mechanism involved, for all three of these activities are destroyed when an extract is heated at 65°C. Moreover, an extract prepared from washed pneumococci, that is the "incomplete" type of cell extract which by itself exhibits none of these reactions, may be activated in each instance by the addition of the same substances, meat infusion or yeast extract.

As already pointed out in the first paper of this series (1), the peroxide-forming activity varies with different strains of pneumococci. These differences are reflected in the activity of the bacterial extracts not only in the peroxide-forming function, but also in the other oxidation-reduction processes described (2–4), including the hemoglobin destruction discussed in this paper. The fact that the sterile extracts possessing the most marked peroxide-forming activity cause the most marked destruction of hemoglobin may be interpreted as further evidence that the same system is involved in these two functions of the extracts.

In the destruction of oxyhemoglobin, as in the oxidation of pneumococcus hemotoxin by sterile extracts, the type of reaction induced

represents one in which a substance, not itself easily oxidized by molecular oxygen, is oxidized through the agency of some product formed by the union of molecular oxygen with the reactive substance in the extract. It may be recalled that an active pneumococcus extract prepared from unwashed bacterial cells, consists of two factors, each of which is separably inactive. One of these factors represents certain constituents of the bacterial cell which are heat-labile and are present in saline extracts of washed pneumococci. Extracts of this type are "incomplete" and non-reactive with molecular oxygen. The other constituents which are essential to the complete oxidation-reduction system may be furnished from sources other than those of bacterial origin. Meat infusion and yeast extract contain thermostable substances which, although themselves inactive, serve to complement the potentially active but otherwise deficient system of the washed cell. With these distinctions in mind, the process involving the change of oxyhemoglobin by an active reduced extract of pneumococcus may be schematically represented as consisting of the following intermediary reactions. If C be allowed to represent the thermolabile cell constituents including the bacterial enzymes and RH the easily oxidizable thermostable substances, then the active system in the broth extract in the reduced state would consist of [C + RH]; and

$$[C + RH] + HbO_2 \longrightarrow ROOH + Hb$$

$$ROOH + Hb \longrightarrow MetHb + \text{other oxidation products.}$$

The first intermediary step involves the deoxygenation of the oxyhemoglobin.[2] That a source of oxygen in the system is essential

[2]It is equally in keeping with the experimental data to interpret the methemoglobin-forming reaction in a manner somewhat more analogous to Conant's interpretation of the action of potassium ferricyanide on hemoglobin solutions. Reduced hemoglobin, oxyhemoglobin, and molecular oxygen are in equilibrium in the system. Upon the addition of reduced pneumococcus extract the molecular oxygen unites with constituents of the extract. The oxidizing agent thus formed oxidizes reduced hemoglobin to methemoglobin. This reaction, by removing either (or both) reduced hemoglobin or molecular oxygen, causes more oxyhemoglobin to dissociate until there are established the final equilibria between the oxidizing agent, reduced hemoglobin, and methemoglobin, and between oxyhemoglobin, reduced hemoglobin, and molecular oxygen.

for the initiation of the reactions is clearly shown by the fact that reduced extract in the presence of reduced hemoglobin does not form methemoglobin. The oxygen furnished by the oxyhemoglobin unites with the reactive substance of the extract to form a peroxide (ROOH). This peroxide in turn oxidizes the reduced hemoglobin to methemoglobin. The necessity of oxygen for the initiation of the reaction and the demonstration of hydrogen peroxide in extracts which under the influence of ordinary oxygen have changed from the reduced to the oxidized form, suggest that hydrogen peroxide as such may be the active agent in the oxidation of hemoglobin. However, the fact that hydrogen peroxide may be detected as an end-product in oxidized pneumococcus extract does not necessarily imply that other active, but transient products may not be formed in the earlier stages of the reaction. Indeed, there are reasons to believe that the actual agent in the oxidation of hemoglobin under these conditions may be a higher, organic peroxide, a precursor of hydrogen peroxide. One reason for this view is the fact that if blood is present during the oxidation of a reduced extract, the active substance that is formed is wholly unaffected by blood catalase, transforming hemoglobin in the presence as well as in the absence of this enzyme. On the other hand, if pneumococcus extract is completely oxidized before it is added to oxyhemoglobin, methemoglobin is formed only in the absence of catalase. In this respect an oxidized extract behaves toward hemoglobin merely as does a dilute solution of hydrogen peroxide. These differences are brought out in those experiments in which the comparative action of a reduced and oxidized extract on oxyhemoglobin was studied in the presence and absence of catalase.

The conception that methemoglobin formation by pneumococcus involves the preliminary deoxygenation of oxyhemoglobin was suggested by Cole (10). The present study supports this interpretation and is in accord with the recently published evidence (Conant (14)) that the transformation of hemoglobin to methemoglobin is a true oxidation in the electronic sense, while the conversion of hemoglobin to oxyhemoglobin is merely an oxygenation. Conant interprets the transformation of oxyhemoglobin to methemoglobin by the action of ferricyanide as a reaction involving the *oxidation* of previously

deoxygenated hemoglobin. The actual oxidation process, which gives methemoglobin, Conant believes to consist in the oxidation of the ferrous iron of deoxygenated hemoglobin to the ferric form. This reaction may be brought about by a number of oxidizing agents. The present study suggests that the oxidizing agent in the case of pneumococcus is a peroxide of bacterial origin.

From the experimental evidence available, it is inferred that in the formation of methemoglobin by pneumococcus, the initial reaction involves the deoxygenation of oxyhemoglobin and the union of the free oxygen with some autoxidizable substance of the cell and that a peroxide thus formed serves as the actual oxidizing agent in the conversion of the hemoglobin to methemoglobin.

SUMMARY.

1. Sterile broth extracts of unwashed pneumococci actively destroy hemoglobin to methemoglobin and lower degradation products.

2. Sterile saline extracts of washed pneumococci do not by themselves form methemoglobin; extracts of this type may be completed or activated by the addition of certain complementary substances such as meat infusion and yeast extract.

3. The hemoglobin-destroying activity of pneumococcus extract is lost by exposure to 65°C. for 10 minutes.

4. The properties of an extract upon which these blood changes depend are related to other known oxidation-reduction functions of the same extract.

5. Oxyhemoglobin is converted to methemoglobin only by cell extracts in the reduced form; completely oxidized extracts are inactive in the presence of blood. The action of hydrogen peroxide and the influence of blood catalase on these reactions are discussed.

6. During the reaction between oxyhemoglobin and pneumococcus extract oxygen is consumed.

7. The mechanism of methemoglobin formation by pneumococcus is interpreted as an oxidation process in which deoxygenation of oxyhemoglobin and peroxide formation occur as intermediary reactions. The active agent of the oxidation of hemoglobin is considered to be a peroxide of bacterial origin.

BIBLIOGRAPHY.

1. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 347.
2. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 357.
3. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 543.
4. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 745.
5. Heidelberger, M., *J. Biol. Chem.*, 1922, liii, 31.
6. Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.
7. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924 (in press).
8. Stadie, W. C., *J. Biol. Chem.*, 1920, xli, 237.
9. Butterfield, E. E., and Peabody, F. W., *J. Exp. Med.*, 1913, xvii, 587.
10. Cole, R., *J. Exp. Med.*, 1914, xx, 363.
11. Stadie, W. C., *J. Exp. Med.*, 1921, xxxiii, 627.
12. Schnabel, A., *Z. Hyg. u. Infectionskrankh.*, 1921, xciii, 175.
13. Meyerhof, O., *The Harvey Lectures*, 1923.
14. Conant, J. B., *J. Biol. Chem.*, 1923, lvii, 401.
15. M'Leod, J. W., and Gordon, J., *J. Path. and Bact.*, 1922, xxv, 139.